The Effect of Topical Neuromodulatory Medications on Orofacial Tissue *In Vitro*

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Abstract

Systemic neuromodulatory medications (NMs) are the conventional management modality of neuropathic orofacial pain (NOP). Topical administration of NMs such as amitriptyline, carbamazepine and gabapentin have been reported to show promising results when used with NOP, however, the effect of exposure of oral mucosa and skin to topical NMs has not been examined and is therefore unlicensed. The aims of the research contained within this thesis were to: a) investigate the effects of the aforementioned NMs on cell lines and 3D tissue relevant to the orofacial tissue *in vitro;* b) conduct a preliminary study using RNA extracted from human gingival tissue to investigate whether the potential therapeutic targets for these NMs were expressed.

Cellular viability was measured using alamarBlue®, testing the effects of NMs on monolayer cell culture (2D): human skin and oral keratinocytes and mouse fibroblasts. Effects on cell counts were investigated by a CCK-8 assay. Morphological changes and cytokine expression were investigated using scanning electron microscopy and antibody array, respectively. Human gingival tissue biopsy was used to investigate the expression of receptors at the mRNA level using PCR.

Amitriptyline exposure was found to decrease cellular viability and count, along with morphological changes as opposed to carbamazepine and gabapentin which had little demonstrable effects. Amitriptyline's cytotoxic effect was confirmed using 3D oral mucosa models. Amitriptyline's effect on 3D models was then further examined using immunohistochemistry (H&E, anti-caspase 3 antibodies) and immunofluorescence (anti-amitriptyline antibody). In the 3D model, amitriptyline caused apoptosis after repeated exposure, but was able to traverse tissue barriers, which was also confirmed by High Performance Liquid Chromatography (HPLC).

The results of preliminary genetic investigations suggested the expression of the most receptors analysed, this suggests that further studies are required to investigate the efficacy of the topical route as a treatment for NOP.

The overall results demonstrate amitriptyline toxicity and avoiding topical amitriptyline is therefore likely to be advisable. Carbamazepine and gabapentin exposure were less harmful and possibly more suitable topical choices, but further studies need to be conducted.

Certificate of approval

I confirm that, to the best of my knowledge, this thesis is from the student's own work and effort, and all other sources of information used have been acknowledged. This thesis has been submitted with my approval.

Signature

Supervisor: Dr Justin Durham

List of abbreviations

AAOP	American Association of orofacial pain
AMI	Amitriptyline
AO	Atypical odontalgia
B.I.D.	Twice a day
BMS	Burning mouth syndrome
CBZ	Carbamazepine
CCK-8	Cell counting kit-8
cDNA	Complementary DNA
cm	Centimetre
CNP	Central neuropathic pain
CNS	Central nervous system
CRPS	Complex regional pain syndrome
dd H2O	Double–distilled water
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMA	European medicines agency
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
GAB	Gabapentin
GABA	Gamma amino-butyric acid
GAPDH	Glyceraldehyde-3’-phosphate dehydrogenase (housekeeping gene)

H	Hours
H&E	Haematoxylin and Eosin
HPLC	High-performance liquid chromatography
IASP	International Association Of Study Of Pain
IC50	Inhibitory concentration that reduced the response to half
IHS	International Headache Society
KSFM	Keratinocyte-Serum Free Media
LD_{50}	Lethal dose 50%
LDH	Lactate dehydrogenase assay
LNP	localized neuropathic pain
mRNA	Messenger RNA
mM	Millimolar
Min	Minutes
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NICE	National Institute Of Health And Care Excellency
nm	Nanometres
NMDA	N-methyl D-aspartate
NMs	Neuromodulatory medications
NOP	Neuropathic orofacial pain
NP	Neuropathic pain
OFP	Orofacial pain
PBS	phosphate buffered saline
PDAP	Persistent dento alveolar pain
PNP	Peripheral neuropathic pain
PHN	Postherpetic neuralgia

PNS	Peripheral nervous system
RDC	Research diagnostic criteria
PCR	polymerase chain reaction
SE	Standard error of the mean
SEM	Scanning electron microscope
SSNRIs	Selective serotonin and noradrenaline reuptake inhibitors
T.I.D.	Three times a day
TCA	Tricyclic antidepressants
TMD	Temporomandibular disorders
TN	Trigeminal neuralgia
TRPV1	Transient receptor potential V1
TTX	Tetrodotoxin
µl	Microlitre
µM	Micromolar
µm	Micrometre
v/v	Volume/volume
w/v	Weight/volume

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1.1 Pain

Pain is a complicated biopsychosocial experience, starts as a noxious stimulus, transduced and transmitted to the sensory cortex (nociception). The sensation and recognition of pain in the sensory cortex is then processed and reported as the pain experience (Renton, 2015).

The perceptual biological activity of pain, is managed by peripheral and central complex network of neurons and transmitters (Basbaum and Woolf, 1999; Basbaum *et al.*, 2009). Noxious stimuli transduction starts as molecular and signalling interaction at the peripheral nerve endings. These nerve endings express various receptors and ion channels with major role in pain pathophysiology. With increased understanding of these peripheral activities, peripheral receptors and ion channels have been raised as possible targets for topical treatment in chronic pain management such as neuropathic pain (NP). The topical modality was found feasible and promising as a treatment of NP, with fast action and low side effect profile (Heir *et al.*, 2008; Nasri-Heir *et al.*, 2013; Zur, 2014).

This chapter describes the various types and mechanisms of pain in the orofacial region with a more in-depth discussion of neuropathic pain (NP) and the potential mechanisms and role of topical treatment in this type of orofacial pain.

1.1.1 Pain definition and types

Pain is a subjective experience, which impairs the patient's life through emotional, sensory and behavioural impacts. It is defined by The International Association for the Study of Pain (IASP) as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" (Merskey and Bogduk, 2012).

According to the duration of pain it can either be defined as acute or chronic. Acute pain begins quickly as seen after injury or surgery and lasts for short

duration. It is considered an adaptive, protective, mechanism until healing is completed (De Leeuw and Klasser, 2013). Chronic pain according to IASP is pain persisting for 3 months or more, that is beyond the normal healing period. Persistent pain may cause neuroplastic and behavioural changes, which may then make the pain more difficult to manage (Flor, 2003; Merskey and Bogduk, 2012; De Leeuw and Klasser, 2013).

Pain may be classified according to aetiology and sequelae into three main types (Costigan *et al.*, 2009; Woolf, 2010):

1. Nociceptive pain: acting as a protective system, which begins operating in response to primary sensory neurons' exposure to different noxious stimuli.

2. Adaptive pain sensation: develops in the healing period following tissue injury during which even innocuous stimuli produce pain as a protective mechanism from further damage.

3. Maladaptation or dysfunction of nervous system for example neuropathic pain.

1.1.2 Mechanism and pathway of pain in the orofacial region

The trigeminal nerve, through its three divisions the ophthalmic, maxillary and mandibular, represents the main conduit for the pain signal conduction in the orofacial region Figure 1.1, (Sessle, 2000). The very first aspect of pain is nociception where external noxious stimulation of the specialized nerve endings of primary afferent neurons (nociceptors) in the trigeminal system induces depolarization and action potentials in the nociceptors (the afferent nerve fibre′s ending). The impulse then propagates to the trigeminal ganglia where the cell bodies of primary afferent neurons are located (Basbaum and Woolf, 1999; Julius and Basbaum, 2001; De Leeuw and Klasser, 2013).

Nociceptors are defined by IASP as "*a high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and*

encoding noxious stimuli" (Merskey and Bogduk, 2012). There are two types of nociceptors responsible for pain transduction: the medium diameter lightly myelinated Aδ (A-delta) fibres which rapidly conduct impulses and are responsible for the first pain response. The second type of nociceptors are unmyelinated small diameter C-fibres which are characterized by slow conduction of impulses and responsible for second pain response (Julius and Basbaum, 2001). Both C-fibres and Aδ fibres respond with high threshold to the noxious stimuli provoking nociceptive pain dull and sharp respectively (harmful sensation). The C-fibres are polymodal, therefore responding to a range of stimuli including chemical, thermal and mechanical. The Aδ fibres, however, mainly respond to thermal and mechanical stimuli (Julius and Basbaum, 2001; Woolf and Ma, 2007; Dubin and Patapoutian, 2010). Different tissues′ receptors may respond differently for example in teeth where any stimulus to pulp is considered noxious and provokes pain (Julius and Basbaum, 2001). The primary afferent neurons travel into and through the trigeminal ganglion with very little crossover entering into the brainstem and the Trigeminal Nuclear Complex (TNC) through the sensory nerve root from the trigeminal ganglion. Within the TNC, they synapse with second-order neurons in the sub nucleus caudalis (SN caudalis). Along with SN oralis and SN interpolaris, the SN caudalis forms the spinal sensory nucleus of the trigeminal nuclear complex. The two types of second-order neurons: nociceptive–specific (NS) and wide dynamic range neurons (WDR) transfer pain impulses to the higher brain centres through the antero-lateral spinothalamic tract, feeding back to the other two trigeminal sensory nuclei, the Mesencephalic and Principal nuclei, which have a major role in proprioception.

SN caudalis transfers pain impulses to the higher brain centres for perception and response by one of two pathways according to the pain type. Sharp and fast pain impulses transfer by ascending WDR neurons through the neospinothalamic tract directly to the thalamus and then to the cortex. A descending impulse is then immediately produced to the motor neurons presenting as muscle contraction as in flinch response and blinking.

Dull pain transfers by NS neurons through the paleospinothalamic tract and trough reticular formation before it reaches thalamus, cortex, limbic structure

and hypothalamus for perception and response (Okeson, 2005; De Leeuw and Klasser, 2013).

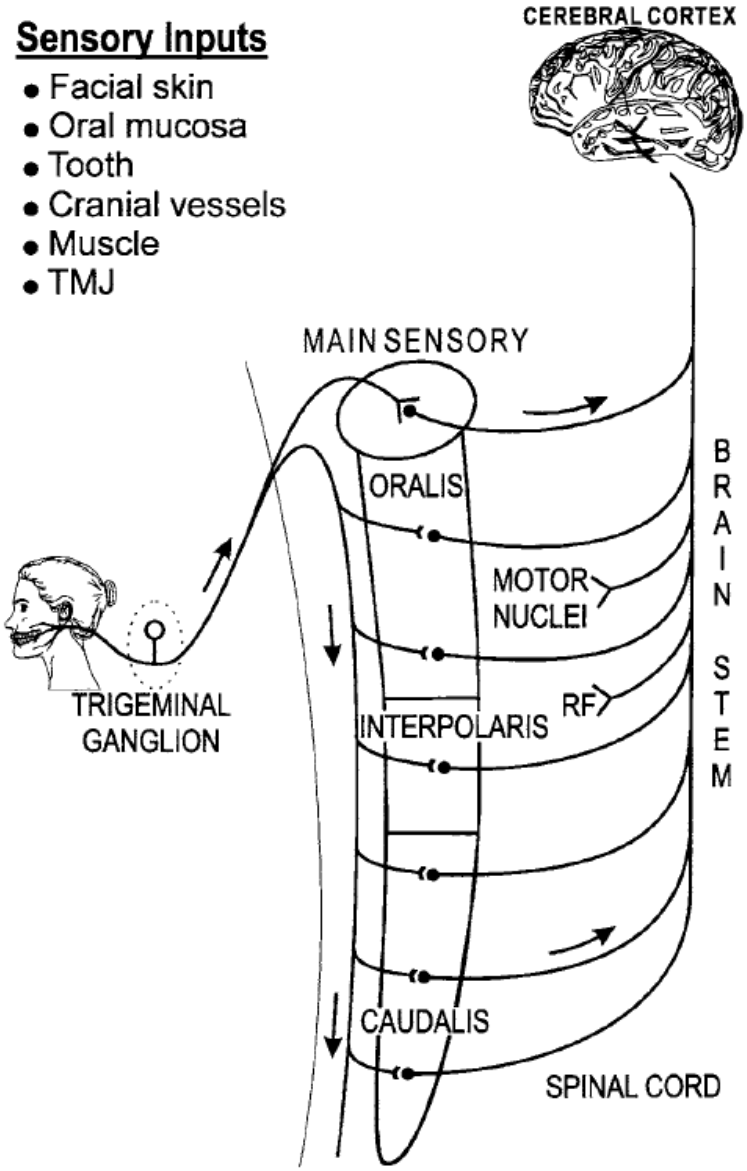


Figure 1.1: Major pain pathway in orofacial region through trigeminal nerve. Reproduced from Sessle (2000) with permission, Appendix A. 1.

1.1.3 Sensitization and neuroplasticity

Inflammation following trauma or surgery in the orofacial region causes the release of inflammatory mediators, creating the so-called “inflammatory soup” which includes inflammatory mediators such as prostaglandins and bradykinin (Verdu *et al.*, 2008). These mediators induce changes in the nociceptors′ characteristics and can increase nociceptive transmission centrally by a number of mechanisms including: lowering the stimulation threshold, increasing the likelihood of spontaneous activity, and recruitment of silent nociceptors. This process is known as peripheral sensitization and it is defined by IASP as an “*increased responsiveness and reduced threshold of nociceptive neurons in the periphery to the stimulation of their receptive fields*” (Merskey and Bogduk, 2012).

Due to the amplified input from peripheral nociceptors central neurons become more active and sensitive with modulation of synaptic excitability and transmitter production/ release in the brain stem. Long lasting excitability and inflammation induce changes in receptors and cytokine expression in addition to the mutation of receptors’ genes (Ji and Strichartz, 2004). IASP defined central sensitization as “*Increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input*” (Petersen-Felix and Curatolo, 2002; Merskey and Bogduk, 2012).

The previously described augmentations in the detection and perception of noxious stimuli and the associated chain of peripheral and central modulation of nociception begins as reversible changes but may become permanent over time and thereby cause chronic pain. Concomitant to the previously described changes, glia and microglia cells have also been implicated (Scholz and Woolf, 2007), both peripherally and centrally, in central sensitisation through synthesis and release of various cytokines and mediators that induce or maintain the sensitised state.

The changes explained in the previous paragraphs are part of what is known as neuroplasticity which relates to the ability of nervous tissue to produce different changes in its structure and function in response to both internal and external

stimuli (Petersen-Felix and Curatolo, 2002; Chiang *et al.*, 2011; De Leeuw and Klasser, 2013).

1.2 Neuropathic pain

The IASP has introduced a revised definition of (NP) as "*Pain caused by a lesion or disease of the somatosensory nervous system*" (Treede *et al.*, 2008; Merskey and Bogduk, 2012). Clinically, patients with NP present with one or more types of pain and sensory disturbances:

1. Spontaneous pain (stimulus independent): which occurs either as a paroxysmal shooting, electrical shock like pain or constant continuous burning sensation.
2. Stimulus dependent pain: including allodynia, pain in response to innocuous stimuli, and hyperalgesia which is increased response to noxious stimuli (Jensen *et al.*, 2001; Jensen and Baron, 2003).

Different negative sensations can also present with the pain including paresthesias, hypoesthesia and hypoalgesia; some of which predominate in peripheral or central NP or both (Woolf and Mannion, 1999; Jensen *et al.*, 2001; Jensen and Baron, 2003).

Clinical examination is the key step in diagnosis and assessment of NP. Sensory assessment including: thermal, cold, pinprick and touch, is convenient method for initial diagnosis in chairside examination. The further psychophysiological and more sophisticated assessment could be applied for further confirmation and research purposes (Haanpää *et al.*, 2011). Recently, Finnerup *et al.* (2016) introduced a grading system with sophisticated methods such as QST, and biopsy to give definitive NP diagnosis, however, that is may be acceptable for research centres with available budget and facility but it is rather difficult to apply for every clinical examination and decision including NOP.

1.2.1 Neuropathic pain Types

Neuropathic pain can be subdivided into peripheral and central NP according to the site of the causative disease or lesion.

Central neuropathic pain

IASP defined central neuropathic pain (CNP) as "*Pain caused by a lesion or disease of the central somatosensory nervous system*", associated with central nervous system (CNS) trauma and neurological conditions such as stroke and multiple sclerosis.

Peripheral neuropathic pain

Peripheral neuropathic pain (PNP) can be the result of infection, trauma or toxic chemicals and is defined by IASP as "*pain caused by a lesion or disease of the peripheral somatosensory nervous*" (Dworkin *et al.*, 2003; Costigan *et al.*, 2009). Baron *et al.* (2010) proposed a further subdivision of PNP to into: focal /multifocal neuropathic pain as in post-traumatic neuralgia, postherpetic neuralgia and phantom pain; generalized peripheral neuropathic pain as in drug toxicity and diabetes polyneuropathy.

Focal and multifocal PNP is a more localized entity which appears to have better management results with topical treatment such as lidocaine patches.

Localized neuropathic pain

The term "localized neuropathic pain" (LNP) came into use in response to clinical findings of localized and well circumscribed areas of PNP which were responsive to topical treatment (Dworkin *et al.*, 2007; De Moulin *et al.*, 2014). LNP is defined as a "*type of neuropathic pain that is characterized by consistent and circumscribed area(s) of maximum pain associated with negative or positive sensory signs and/or spontaneous symptoms characteristic of neuropathic pain" (Mick et al., 2011)*.

LNP is thought to be present in more than 60% of all neuropathic pain patients (Mick *et al.*, 2011) and it presents as a part of PNP for example postherpetic neuralgia and diabetic neuropathy with an association with central plasticity (Casale *et al.*, 2014; Casale and Mattia, 2014). LNP is not, however, officially

classified, but is often mentioned whenever topical treatment is suggested in the NP management guidelines (Dworkin *et al.*, 2007; NICE, 2013; De Moulin *et al.*, 2014) or as Finnerup *et al.* (2015) termed LNP the presence of a “local pain generator”.

1.2.2 Mechanisms of neuropathic pain

Different types of NP share similar signs and symptoms of pain independent of their underlying causes and mechanisms. NP mechanisms include multiple changes and modulations, which may contribute peripherally and centrally to NP’s intensity and clinical picture (Finnerup *et al.*, 2007; Baron *et al.*, 2010).

At the peripheral level, a lesion in the primary afferent nerve fibres is considered essential to initiate NP (Baron, 2006). Following this a series of mechanisms will develop peripheral sensitivity and give rise to spontaneous or ectopic pain. The spontaneous pain may be related to increase in expression of voltage-gated sodium channels on both injured and undamaged nerve fibres and at the same time lowering of the action potential threshold of these afferents (Hunt and Mantyh, 2001). Other ion channels, including voltage-gated potassium channels also undergo alteration and lead to increased excitability in primary afferents. Specific receptors, such as the transient receptor potential V1 (TRPV1), respond to thermal stimuli about 41° C under normal physiological conditions (Scholz and Woolf, 2002; Baron *et al.*, 2010). Following injury these thermally responsive receptors tend to become downregulated on the affected neurons and upregulated on unaffected ones. This phenomenon explains the heat allodynia in which burning sensations may occur in response to normal body temperature and/or endogenous signalling. Cold allodynia has also, however, been reported in specific cases of NP which may result from upregulation of definite cold receptors (TRPM8) (Finnerup *et al.*, 2007; Zagury *et al.*, 2011). The inflammatory process following nerve injury and activation of microglia also contribute to the chronicity of NP by releasing inflammatory mediators and increasing sensitivity at both central and peripheral levels (Finnerup *et al.*, 2007; Baron *et al.*, 2010).

Several changes may develop centrally as a normal consequence of exaggerated peripheral afferent input thereby leading to central sensitization.

These changes at central level may start as secondary hyperalgesia and allodynia are transduced by mechanosensitive receptors (Aβ fibres and Aδ fibres). The central changes may also lead to increase of synaptic strength through synaptic changes. At the presynaptic level there are modulations in synthesis of neurotransmitters and neuromodulators, and increase in calcium channel expression. At the postsynaptic connection there are increases in ion channel production, enhancing receptor density and phosphorylation of N-methyl-D-aspartate (NMDA) subunits. Increased input from peripheral sensitivity may induce serial events within inhibitory pathways such as degeneration of the inhibitory interneurons in medullary (spinal trigeminal nucleus caudalis) and/or dorsal horn regions, resulting in exaggeration of postsynaptic sensitivity. The inhibitory transmitters accompanying the descending pathway from the cortex and/or hypothalamus may be also shifted from expected inhibitory action to more facilitation action (Campbell and Meyer, 2006; Costigan *et al.*, 2009).

1.3 Orofacial pain

Orofacial pain (OFP) is "*pain associated with the hard and soft tissue of the head, face and neck*" (De Leeuw and Klasser, 2013). It therefore occurs within the distribution of the trigeminal nerve sensory supply (Lavigne *et al.*, 2005). Several studies investigating orofacial pain recognise its multidimensional nature and consider it a biopsychosocial phenomenon (Robert *et al.*, 2008; Hargreaves, 2011). Many studies have attempted to identify the prevalence of OFP in the general population with estimates varying between 7% and 25% in different study populations (Macfarlane *et al.*, 2002; Aggarwal *et al.*, 2006; Benoliel *et al.*, 2008).

The diagnosis of OFP can present difficulties to the specialist and generalist alike as many disorders share similar signs and symptoms with comorbidities to psychological profiles which can contribute, and add, to the complexity of the presenting pain (Durham *et al.*, 2007; Aggarwal *et al.*, 2011; Hargreaves, 2011; De Leeuw and Klasser, 2013). Some of the diagnostic challenges can be related to the complex innervation of orofacial region and different, highly specialized, tissues that can make it difficult sometimes to identify the origin of the pain in addition to any comorbid psychosocial factors that may complicate the presenting picture further (Benoliel *et al.*, 2008; Renton *et al.*, 2012a).

1.3.1 Classification of orofacial pain

The complex nature of OFP conditions makes an easily applicable classification difficult. Several attempts have been made to develop a robust and applicable classification for OFP based on aetiology, anatomy and duration of pain, but with less professional satisfaction regarding research and or clinical applications of the resultant classification or criteria (Renton *et al.*, 2012a). The main three classifications: the International Headache Society's classification of headache disorders (IHS) in 2013 (Silberstein *et al.*, 2005; De Leeuw and Klasser, 2013; IHS, 2013); the disorder-specific Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) in 2013; the more generic IASP classification in 1994 (Dworkin and LeResche, 1992; Okeson, 2008; Merskey and Bogduk, 2012; Renton *et al.*, 2012a; De Leeuw and Klasser, 2013; Schiffman *et al.*, 2014). The American Academy of Orofacial Pain's (AAOP)

classification tries to utilise the other preceding classifications to some extent and present an amalgamation of them (De Leeuw and Klasser, 2013)

The AAOP guidelines for orofacial pain diagnosis and management (De Leeuw and Klasser, 2013) subdivides OFP into the following main categories:

1. Vascular and nonvascular Intracranial pain disorders: many of these are associated with life-threatening conditions such as neoplasm, hematoma.
2. Primary headache disorders: common presentations include migraine and tension headache.
3. Neuropathic pain disorders: including the episodic and continuous types.
4. Intraoral pain disorders: the commonest form of orofacial pain.
5. Temporomandibular disorders: main non dental cause of pain
6. Cervical pain disorders: mostly related to the cervical spine.
7. Extracranial and systemic causes of orofacial pain: mostly referred pains from associated structures such as the ears, nose and salivary glands.

The most frequently presenting pain conditions in primary care are intraoral disorders, musculoskeletal disorders and NP (Scully and Felix, 2006; Bell *et al.*, 2008; Aggarwal *et al.*, 2011).

The most common orofacial pain category is intraoral disorders which includes odontogenic and non-odontogenic diseases affecting oral hard tissue including bone and tooth structure and/or oral soft tissues including the dental pulp, gingivae, lining mucosa, periodontal tissues, tongue and salivary glands (McNeil *et al.*, 2008). After intraoral pain, temporomandibular disorders (TMD) are the next common category of chronic orofacial pain. Temporomandibular disorders (TMDs) term is defined by the AAOP as "*encompass a group of musculoskeletal and neuromuscular conditions that involve the TMJs, masticatory muscles and all associated tissues*" (De Leeuw and Klasser, 2013)

The previous two types of pain are recognised by nervous system nociceptors and categorized into protective and adaptive pain types, with TMDs having the potential to become a maladaptive persisting sensation through a process of central sensitisation (Sarlani and Greenspan, 2003). One maladaptive sensation that is likely to be maladaptive from its inception is NP. This

“neuropathic pain” can impact on almost all aspects of the patient’s life (Treede *et al.*, 2008; Costigan *et al.*, 2009).

1.3.2 Types of neuropathic orofacial pain

Among various types of NOP, the following groups of NOP have been found to be treatable by topical neuromodulatory medications (NMs): trigeminal neuralgia (TN), glossopharyngeal neuralgia, postherpetic neuralgia (PHN), burning mouth syndrome (BMS), atypical facial pain, persistent dento alveolar pain (PDAP) and complex regional pain syndrome (CRPS), (Argoff, 2003; Bennett, 2004; Zakrzewska, 2010; Nixdorf *et al.*, 2012a; De Leeuw and Klasser, 2013).

a- Trigeminal neuralgia

Trigeminal neuralgia presents as a recurrent, severe, stabbing, mostly unilateral pain within the distribution of the trigeminal nerve often initiated by contact with a trigger zone (Durham, 2011). Recently, IHS classification has divided TN according to its aetiology and clinical picture into “classical trigeminal neuralgia, purely paroxysmal and *Classical trigeminal neuralgia with concomitant persistent facial pain*”, (IHS, 2013). Trigeminal neuralgia is mostly diagnosed as unilateral paroxysmal pain associated with either the 2nd or 3rd divisions of the trigeminal nerve, often affecting females in their seventh decade without identifiable cause (Lopes *et al.*, 2002; Zakrzewska, 2010; De Leeuw and Klasser, 2013). It is a rare disease as reported by different epidemiological studies with prevalence range about 4 – 6 per 100,000 of population. However, higher results reported in United Kingdom and Netherlands studies, about 26 - 28 incidences per 100,000 of population. These wide variations in prevalence may be related to the criteria have been used in the diagnosis (Katusic *et al.*, 1990; Hall *et al.*, 2006; Dieleman *et al.*, 2008; Zakrzewska, 2010).

b- Glossopharyngeal neuralgia

Glossopharyngeal neuralgia is defined by the IASP as a “*sudden severe brief stabbing recurrent pains in the distribution of the glossopharyngeal nerve”* (Merskey and Bogduk, 2012*)*. It is a rare condition affecting the 9th cranial nerve with severe, stabbing episodic pain presenting in the ear, base of tongue and beneath the angle of the mandible. Similar to TN, it predominantly presents in

elderly females but is reportedly less prevalent than TN presenting in 0.8 per 100,000 of population (Katusic *et al.*, 1991; Zakrzewska, 2010).

c- Postherpetic neuralgia

Postherpetic neuralgia (PHN) is a constant burning pain which can also present with stabbing sensation. These symptoms result from neuronal destruction both peripherally and centrally (at dorsal horn) as a complication of latent varicella-zoster virus (shingles). After the thoracic region, the orofacial region is the next most frequently affected region, especially the region innervated by ophthalmic division of the trigeminal nerve (Sayanlar *et al.*, 2012). PHN commonly affects elderly people (≥ 65), in which 50% of elderly may be affected especially females (Bennett, 2004; Merskey and Bogduk, 2012; De Leeuw and Klasser, 2013)

d- Burning mouth syndrome (BMS)

BMS is subdivided into primary and secondary BMS. Sardella *et al.* (2006) defined primary BMS as "*a common dysesthesia described as burning sensation in the oral mucosa occurring in the absence of clinically apparent mucosal abnormality or laboratory finding and often perceived as painful*". Secondary BMS is a burning sensation secondary to precipitating disorders such as anaemia, diabetes, specific medications (Jääskeläinen, 2012). BMS patients commonly present complaining of pain in the anterior tongue, hard palate and lower lip, and occasionally the whole mouth is involved (Grushka *et al.*, 2003). Concomitant symptoms of xerostomia and taste disturbance (dysgeusia), especially bitter and metallic tastes are also commonly reported in BMS (Grushka *et al.*, 2003; Amos *et al.*, 2011). Epidemiological studies report a wide variation in the prevalence of BMS ranging from 0.7% to 15% (Zakrzewska *et al.*, 2005; López-Jornet *et al.*, 2010). This is likely due to the lack of accepted diagnostic criteria and other variations in method between the studies (Bergdahl and Bergdahl, 1999; Scala *et al.*, 2003; Zakrzewska *et al.*, 2005). Like most other NOP, BMS predominantly presents in older, postmenopausal females (Lavigne *et al.*, 2005; Ko *et al.*, 2012). There is uncertainty over the pathophysiology of primary BMS, however, the human studies and treatment trials suggest it is most likely to neuropathic in origin (Lauria *et al.*, 2005; Eliav

et al., 2007; Yilmaz *et al.*, 2007; Zakrzewska, 2010; de Tommaso *et al.*, 2011; De Leeuw and Klasser, 2013).

e- Persistent dento-alveolar pain

Chronic idiopathic intraoral pain without definitive pathology has been explained, described and classified as different entities or as pseudonyms such as: phantom tooth pain, atypical facial pain, atypical odontalgia, atypical trigeminal neuropathic pain or idiopathic continuous neuropathic pain (Marbach, 1978; Woda *et al.*, 2005; Baad-Hansen, 2008; De Leeuw and Klasser, 2013). One of the classifications introduced by Woda *et al.* (2005) sought to differentiate orofacial idiopathic pains into atypical facial pain (AFP), atypical odontalgia (AO), BMS and TMD. According to anatomical division AFP is a term generally used to describe a group of idiopathic facial pain complaints which are persistent and may be confusing to classify and manage, whilst atypical odontalgia (AO) is a potentially related condition that is usually described as persistent tooth pain often without any objective signs of clinical and/or radiological pathology or known cause (De Leeuw and Klasser, 2013). More recently Nixdorf *et al.* (2012a) reclassified AO as persistent dento- alveolar pain disorder (PDAP). Benoliel *et al.* (2012b) introduced a broad nomenclature for orofacial neuropathy as “painful posttraumatic trigeminal neuropathy” including both facial and oral constant pain and sensation discrepancy. Benoliel’s proposed criteria was adopted in IHS classification (IHS, 2013), while atypical odontalgia description for continuous idiopathic trigeminal pain within dental region, was listed in AAOP classification (De Leeuw and Klasser, 2013).

The reclassification of AO into PDAP aimed to simplify the diagnosis of this chronic orofacial pain and allow it to be categorised in an ontological fashion with the ultimate aim of advancing the management of this subtype of NOP.

The prevalence of PDAP is reported to range from 2 to 6% (Rodríguez-Lozano et al., 2010). PADP may arise after dental intervention involving deafferentation injuries such as endodontic treatment, extraction and even following local anaesthetic injection. Other studies provide information on the incidence of PDAP after implant surgery, surgical endodontic treatment and 3rd molar

extraction (Vickers and Cousins, 2000; Melis *et al.*, 2003; Bennett, 2004; Epstein *et al.*, 2007; Rodríguez-Lozano *et al.*, 2010).

f- Complex regional pain syndrome (CRPS)

CRPS is a continuous burning NP, characterised by swelling and changes in blood flow in addition to allodynia and hyperalgesia of the affected area (Merskey and Bogduk, 2012). The pathophysiology is still unclear but central and peripheral NP mechanisms in addition to immunological, inflammatory causes subdivide the CPRS into I and II types (De Leeuw and Klasser, 2013). Reported cases in the orofacial region are fewer than elsewhere in the body (de Mos *et al.*, 2007; Nixdorf *et al.*, 2012b).

1.4 Treatment of neuropathic orofacial pain

Apart from diversity in diagnosis, treatment with such neuronal system complexity and serial cascading pathophysiology is difficult and challenging all over the body not only in orofacial region (O'Connor, 2009). This is especially true when the patient cohort presenting can be elderly with many comorbid health problems. NP presenting in the orofacial region, as opposed to elsewhere in the body, can be more complex to diagnose due to the need to exclude many more cofactors in order to make a diagnosis and there are limited criteria and tools to facilitate this process (Truelove, 2004; McDermott *et al.*, 2006). Absence of clinical and or radiological lesions limits current treatment to choosing between pharmacological options, except in trigeminal neuralgia and surgical trauma cases. These pharmacological options are characterized by a limited evidence-base for their use in NOP and generally are subject to decreased patient adherence to prescribed regimens due to the medication side-effect profile (Finnerup *et al.*, 2015).

1.4.1 Types of treatment

The treatment of NOP can be difficult for two main reasons: 1) the conflicting evidence base on its management; 2) the pain can be resistant to the usual methods of pain relief (Gilron *et al.*, 2006; de Leon-Casasola, 2007; O'Connor, 2009).

The treatment modalities for NOP relate to the type and site of the lesion and/or the disease or disorder affecting the somatosensory nervous tissue.

1.4.2 Surgical management

Surgical management is predominately for the various macroscopic injuries caused to the trigeminal nerve such as those caused through trauma, wisdom tooth removal and vascular compression in TN. It is not a compulsory choice for all cases and depends on careful assessment and examination of the patient, the cause of their injury, the duration of their injury, their response to medication and their perspectives on surgery (Lopez *et al.*, 2004; Robinson *et al.*, 2004; Renton *et al.*, 2012b). In case of trigeminal neuralgia, the best results were found by either nerve decompression or palliative care destruction procedures,

with chance of recurrence and side effect such as numbness as in palliative care destruction (Zakrzewska and Linskey, 2014).

1.4.3 Pharmacological management

Pharmacological management is often the treatment of choice currently in NOP. Several types of microscopic nerve ending damage can occur following infection, systemic disease and deafferentation; and attempts have been made to manage this type of pain with different types, routes, and dosages of medications (Lewis *et al.*, 2007; Woda and Salter, 2008) which mostly attempt neuromodulation.

A reference guide (Figure 1.2) for NP treatment has been introduced by the National Institute for health and Care Excellence (NICE). In this guideline, first line of treatment are tricyclic antidepressants (eg: amitriptyline) and anticonvulsants (eg: pregabalin). The second choice would be interchange between previous two groups when intolerance of side effect or low effectivity is reported. A third line of opioid and topical capsaicin is also advised in specific conditions (NICE, 2013). Other studies have investigated different systemic medications including N-methyl-D-aspartate (NMDA) antagonist such as ketamine, and anticonvulsants such as carbamazepine in TN (Attal *et al.*, 2010; Zakrzewska, 2010). Due to the side effect profile of most drugs used systemically in management of NOP recent evidence has begun to emerge about the efficacy of topical NMs either alone or in conjunction with other systemic medications (Lewis *et al.*, 2007; Heir *et al.*, 2008).

The side effects of systemic medications and the mechanism of action are listed in Table 1.1. The recommended type of treatment always has been judged by side effect profile alongside level of pain relief. The side effects of systemic medications and the rapid onset of action of topical medications have encouraged many researchers to trial the topical administration of neuromodulatory agents for the management of NP in the orofacial region and elsewhere in the body.

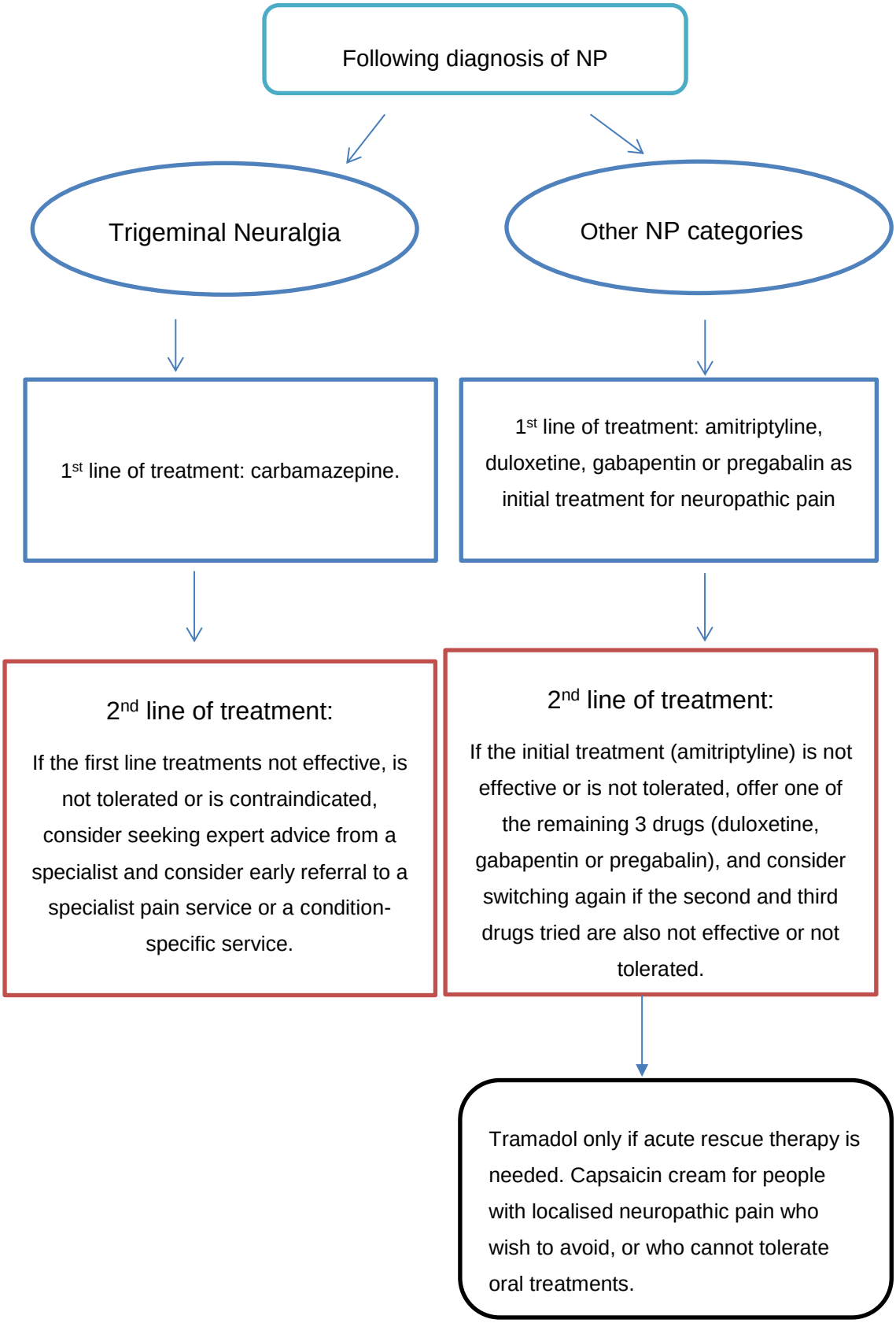


Figure 1.2: NICE guidelines for NP treatment including three main lines of pharmacological treatment (NICE, 2013).

Medications	Mode of action	Major side effects	Precautions
Tricyclic antidepressants (TCA)			
Amitriptyline Nortriptyline Desipramine	Inhibition of reuptake of serotonin and/or noradrenaline, block of sodium, calcium, and potassium channels, anticholinergic, weak NMDA antagonism.	Sedation, dry mouth, blurred vision, urinary retention, weight gain.	Cardiac disease, glaucoma, seizure, suicide risk, concomitant use of tramadol.
Selective serotonin and noradrenaline reuptake inhibitors (SSNRIs)			
Duloxetine	Inhibition of both serotonin and noradrenaline reuptake	Nausea	Hepatic dysfunction, renal insufficiency, alcohol abuse, concomitant use of tramadol
Venlafaxine	Inhibition of both serotonin and noradrenaline reuptake	Nausea	Cardiac disease, concomitant use of tramadol, withdrawal syndrome with abrupt discontinuation.
Calcium channel α2-δ ligands			
Gabapentin Pregabalin	Decrease release of glutamate, noradrenaline and substance P, with ligand on α2-δ sub unit of voltage gated calcium channel. Still some uncertainty about their exact mechanism of action.	Sedation, dizziness, peripheral oedema.	Renal insufficiency
Opioid agonists			
Morphine Oxycodone Methadone Levorphanol	μ-receptor agonism(oxycodone also causes κ-receptor antagonism)	Nausea/vomiting, constipation, dizziness	History of substance abuse, suicide risk, driving impairment
Tramadol	μ-receptor agonism, inhibition of noradrenaline and serotonin reuptake	Nausea/vomiting, constipation, dizziness	History of substance abuse, suicide risk, driving impairment, concomitant use of SSNRI, TCA

Table 1.1: List of systemic medications, mode of action and side effects administered in NP treatment, adapted from Baron *et al.* (2010), Appendix A.2.

1.4.4 Topical treatment

Bar Lignocaine and Capsaicin, low power, uncontrolled, clinical trials and case reports form the main source of evidence regarding the efficacy of topical treatment in NP. This lack of high-quality evidence is the reason why most management guidelines for NP, exclude these compounded topical medications. Compounded topical treatment is, however, increasingly used as an off-label treatment, prescribed and prepared all over the world using various concentrations and types of neuromodulatory agents used as systemic treatments of NP.

Management of NP is in itself a process of trial and error as guidelines contain several different suggestions of systemic antidepressant, anticonvulsant and opioid medications in addition to topical and non-pharmacological treatments (Breivik *et al.*, 2006; NICE, 2013). In McDermott *et al.* (2006) cross sectional study of NP, they found that over a third of patients used topical treatment, with the majority (65 %) of those patients using it for over a year. This may be due to the fact that topical administration of neuromodulatory agents often results in a lessened side effect profile than the same systemically administered medication.

The PNP site, both focal and multifocal sites were found to be more responsive in patients using these types of topical compounding, with relieving of various forms of pain and altered sensations associated with NOP (Padilla *et al.*, 2000; Sawynok, 2003; Heir *et al.*, 2008). Except Lignocaine and Capsaicin, which are already approved for topical use in NP (NICE, 2013), most clinical trials of other topical treatment for NP are preliminary, low power, and uncontrolled studies (De Leeuw and Klasser, 2013; Thompson and Brooks, 2015), however, the clinical trials of other possible pharmacological management strategies and agents keep evolving and an accepted trend for prescription and compounding topical therapies has been established as off-label treatment of NOP, Table 1.2, (Padilla *et al.*, 2000; Bramwell, 2010). Topical treatment for NOP includes patches, creams, mouth wash and other carrier mediums and excipients (Watson, 2004; Benoliel *et al.*, 2012a).

The way these topical treatments are applied depends mainly on site of application,

Table 1.3. For extraoral application, topical treatments are applied using gel, cream and for lidocaine and capsaicin there are commercial plaster and patches (Gilron *et al.*, 2006; Maihofner and Heskamp, 2013). Intra oral application depends on the affected site and size; for localised area, gel or Orabase paste can be applied directly or within custom fabricated stent (Haribabu *et al.*, 2013). In case of multifocal site or difficulty in applying the paste as in burning mouth syndrome or when the tongue is affected, mouth wash of a designed treatment can be used. There is no typical or recognised prescription guideline, these topical treatment are prescribed regarding type, concentrations, and frequency depending on the pain severity, type of drug and clinician experience.

Topical treatment	Type of treatment	Site of action	References
Topical anaesthetic	Lidocaine, EMLA, Benzocaine	Sodium channels	(Padilla *et al.*, 2000; Rogers *et al.*, 2006; Jorge *et al.*, 2011)
Vanilloid compound	Capsaicin	TRPV1 channels	(Jackson, 2006; Moore *et al.*, 2010; Jorge *et al.*, 2011)
Antidepressant	Amitriptyline, Doxepin	Sodium channels, NMDA and adenosine receptors	(Epstein *et al.*, 2008; Liebregts, 2011; Kopsky and Keppel Hesselink, 2012)
Sympathomimetic agent	Clonidine	α1- adrenergic receptors	(Merrill, 2004; Jorge *et al.*, 2011)
Anxiolytic	Clonazepam	$GABA_A$ (Type-A γ-aminobutyric acid) receptor	(Gremeau-Richard *et al.*, 2004)
NMDA blocking agent	Ketamine	NMDA receptors	(Lynch *et al.*, 2005b; Ryan *et al.*, 2009)
NSAIDs	Ketoprofen	Interference with prostaglandin production	(Heir *et al.*, 2008)
Anti-convulsant	Gabapentin	Calcium channels subunit (α2δ-1)	(Heir *et al.*, 2008; Hiom *et al.*, 2015)
	Carbamazepine	Sodium channels	(Padilla *et al.*, 2000; Heir *et al.*, 2008)
Anti-spasmodic drugs	Baclofen	GABA receptor	(Padilla *et al.*, 2000)

Table 1.2: This table showing a list of possible topical treatments for neuropathic orofacial pain with possible target receptors and ion channels.

Medications	Concentrations	Carrier media	References
Capsaicin	0.02 % 0.075 % 8 %	Mouth wash Cream Patch	(Silvestre *et al.*, 2012) (Lewis *et al.*, 2007) (Maihofner and Heskamp, 2013)
Amitriptyline	2% 5 %,10 %	Cream Cream	(Lynch *et al.*, 2005a) (Kopsky and Keppel Hesselink, 2012)
Gabapentin	4%	Lecithin organo gel	(Heir *et al.*, 2008)
Carbamazepine	4%	Lecithin organo gel	(Heir *et al.*, 2008)
Doxepin	3.3% 5 mg/ml (0.5%)	Aqueous cream Mouth wash	(McCleane, 2000) (Epstein *et al.*, 2006)
Clonidine	0.2 mg/g (0.02 %) 4%	Cream Gel	(Epstein *et al.*, 1997) (Graff-Radford and Evans, 2003)
Clonazepam	1 mg 0.5 mg	Tab Tab	(Gremeau-Richard *et al.*, 2004) (Amos *et al.*, 2011)
Ketamine	4%, 1% 0.4 %	Lecithin organo gel Gel Mouth wash	(Heir *et al.*, 2008) (Gammaitoni *et al.*, 2000) (Slatkin and Rhiner, 2003)
Lidocaine	4% 5%	Gel Patch	(Graff-Radford and Evans, 2003) (Gilron *et al.*, 2006)
Ketoprofen	4%	Lecithin organo gel	(Heir *et al.*, 2008)

Table 1.3: This table showing the concentrations, carrier medium and application methods of commonly used topical treatments in management of NP.

a. Topical anaesthetic

Topical anaesthetic such as lidocaine is frequently used as topical medication to manage NP, by blocking voltage gated sodium channels on nociceptors (Padilla *et al.*, 2000; Rogers *et al.*, 2006; Jorge *et al.*, 2011). Lidocaine is an amide local anaesthetic usually used as an injection, but can also applied as cream, gel, mouth wash or patches to relieve NOP. Specifically it has been used in PDAP, BMS, cancer NP and PHN with range of concentrations 2%-10% (Padilla *et al.*, 2000; Gilron *et al.*, 2006; Torgerson, 2010; Pachman *et al.*, 2011; Sankar *et al.*, 2011). An Eutectic Mixture of Local Anaesthetics (EMLA) obtained by combining lidocaine 2.5% and prilocaine 2.5% has also been trialled with PDAP, and it is considered safe and effective for pain relief (Vickers *et al.*, 1997; Vickers *et al.*, 1998; McGeeney, 2009). An ester local anaesthetic, Benzocaine, has also been used for treating NOP when incorporated into orabase as a carrier medium in order to treat intraoral pain (Padilla *et al.*, 2000).

b. Vanilloid compound

Capsaicin is the main component in chilli peppers responsible for the hot sensation derived from genus *Capsicum*. It acts on unmyelinated C-fibres peripheral nerve ending and on vanilloid receptors (TRPV1). After repeated application of capsaicin, a constant pain relief may occur as result of: depletion of substance P, defunctionalisation of TRPV1 receptors and potentially nerve degeneration (Jackson, 2006; Moore *et al.*, 2010; Jorge *et al.*, 2011). Although the first contact burning sensation can limit capsaicin application by the patient, it is utilized to manage various NOP like PHN, BMS and macroscopic trigeminal nerve injury. Different concentrations and methods have been used to deliver it as intraoral topical medication such as cream, patches, mouth wash and even chewing gum or hot tabasco sauce to supply the patient with capsaicin and achieve desensitization (Epstein and Marcoe, 1994; Merrill, 2004; Renton and Yilmaz, 2012; Silvestre *et al.*, 2012). Capsaicin is now one of documented management strategies not only for chronic NP pain but also various musculoskeletal diseases (Lorna *et al.*, 2004; McGeeney, 2009). The chilli characteristic of Capsaicin made it less preferable especially in facial area with NP, however, a few studies have demonstrated promising results with minimal

side effects in case of resistant cases and patients who failed to adhere to systemic treatment (Sayanlar *et al.*, 2012; Gaul and Resch, 2015).

c. Antidepressants

Tricyclic antidepressants (TCA) are the most commonly used systemic treatment for managing NP and amitriptyline is the traditional first choice (Namaka *et al.*, 2004). Amitriptyline's effect on pain when used topically is mediated by several different mechanisms including: inhibition of serotonin and noradrenaline reuptake and blockage of sodium channels (de Leon-Casasola, 2007). In low concentrations amitriptyline provides pain relief and patient satisfaction in peripheral NP (Lynch *et al.*, 2005a), but higher concentrations of amitriptyline containing cream can provide more rapid relief of pain (Liebregts, 2011; Kopsky and Keppel Hesselink, 2012). The antidepressant drug Doxepin is also used to manage different NOPs as a singular drug or in combination with capsaicin (McCleane, 2000). Doxepin is also used as a mouth wash in managing BMS (Epstein *et al.*, 2006; Epstein *et al.*, 2008; Torgerson, 2010).

d. Sympathomimetic agent

Several studies explained that the sympathomimetic agent clonidine is a powerful topical modality in treatment of NP. It blocks sympathetically mediated NP (Padilla *et al.*, 2000) and its mechanism of action is to inhibit noradrenaline release and prevent stimulation of α1- receptors on C-fibres (Merrill, 2004; Jorge *et al.*, 2011). It is applied in management of lingual nerve injury, PDAP and in different causes of NP. It is available as patches, cream or gel (Epstein *et al.*, 1997; Graff-Radford and Evans, 2003; Merrill, 2004; Jorge *et al.*, 2011).

e. Clonazepam

Topical clonazepam is now one of the first choice drugs in BMS management (Buchanan and Zakrzewska, 2004; Lavigne *et al.*, 2005; Zakrzewska, 2010; Sankar *et al.*, 2011). Systemic treatment of BMS with clonazepam showed promising results even with low doses (Grushka *et al.*, 1998). In another trial clonazepam has been topically applied through double blind randomized study by Gremeau-Richard *et al.* (2004). They demonstrated that dissolving 1 mg tab of clonazepam orally and wash around the mouth and then expectorating can relieve the burning sensation of BMS with few side effects. Another study

combined the topical mechanism of Gremeau-Richard *et al.* (2004) alongside swallowing the saliva after mouth washing the tablet around the oral cavity (Amos *et al.*, 2011). The disadvantage of this was that side effects have been reported in about 33% of the patients.

f. NMDA- blocking agent

Ketamine is a general anaesthetic drug which has been used widely as an intravenous or intramuscular analgesic for chronic pain conditions including NOP, cancer and non-cancer pain (Mathisen *et al.*, 1995; Bell, 2009; Noppers *et al.*, 2010). It is an NMDA antagonist which can be applied topically to various types of NP as a gel, mouth wash or cream (Lynch *et al.*, 2005b; Ryan *et al.*, 2009). It has shown promising results in pilot study on NOP by Heir *et al.* (2008). They suggested that topical treatment may be beneficial in mild or moderate pain and could be used as combined therapy with systemic prescription. Two reviews on the management of NOP (Padilla *et al.*, 2000; Merrill, 2004), suggested that using ketamine in conjugation with a stent with or without other medications demonstrated accepted results on intraoral pain. However, the chronic use of ketamine should be under taken with caution because of high dose toxicity and drug abuse.

g. Topical NSAIDs

The major benefits of using topical administration of drugs are avoiding first pass metabolism, associated systemic toxicity and side-effects. This is of significant benefit when using NSAIDs as a topical treatment because gastric upset is the main complication of systemic NSAIDS. The NSAIDs mainly inhibit inflammatory cascade through their interference with prostaglandin production and therefore, perhaps unsurprisingly, there are only a few studies investigating their use in NP. Heir *et al.* (2008) applied Ketoprofen with other medications to improve symptoms in patient with different NOP. He reported successful pain relief with topical medications in mild and moderate cases. Epstein *et al.* (2001) declared that using benzydamine hydrochloride mouth wash by patients with mucositis may be helpful in relieving pain related to cancer and cancer treatment, however, Sardella *et al.* (1999) show that benzydamine mouth wash was not more effective than placebo in managing BMS.

h. Anti convulsant and anti-spasmodic drugs

In addition to the previous drugs, anti convulsants and anti-spasmodic medications are also applied topically to manage NOP. Gabapentin is anticonvulsant medication which is a systemic medication prescribed in epilepsy and NP. It is a gamma amino-butyric acid (GABA) analogue, acting at the central and peripheral nervous system by blocking calcium channels and increasing GABA synthesis. Another anticonvulsant is carbamazepine, which is the drug of choice in TN management. The mechanism of action of Carbamazepine is by blocking sodium channels. The anti-spasmodic drug, baclofen, is GABA receptor agonist mainly used to treat spasticity. Several studies have trialled these three medications topically to manage NOP. They demonstrated that pain relief could be achieved when using these medications as a single drug or in combination with other topical ones (Padilla *et al.*, 2000; Merrill, 2004; Lavigne *et al.*, 2005; Heir *et al.*, 2008).

1.5 Neuromodulatory medications

The types and concentrations of NMs included in this research were decided after literature search regarding NOP treatment (Padilla *et al.*, 2000; Sawynok, 2003; Argoff, 2004; Lynch *et al.*, 2005b; Sawynok, 2005; Heir *et al.*, 2008; McCleane, 2008; Bramwell, 2010; Haribabu *et al.*, 2013), and an informal survey of the international members of the University of California, Los Angeles (UCLA) OFP list-serve about the more common compounded medications used in NOP (Vahedifar, 2010). Among types and concentrations, the three types of NMs found frequently employed in the clinical trials are: amitriptyline, carbamazepine and gabapentin. As these were the neuromodulatory agents chosen as the test medications for this thesis, their pharmacology will now be described in depth.

1.5.1 Amitriptyline

Amitriptyline is a tertiary amine tricyclic antidepressant drug (Figure 1.3), (Viola *et al.*, 2000). Amitriptyline antidepressant characteristics arise from its dual activity as a serotonin, and noradrenaline, reuptake inhibitor at the presynaptic site. This accomplished by blocking the transporters, resulting in an increase of the synaptic level of serotonin and noradrenaline (Fishbain, 2000; Fangmann *et al.*, 2008; Richard *et al.*, 2008).

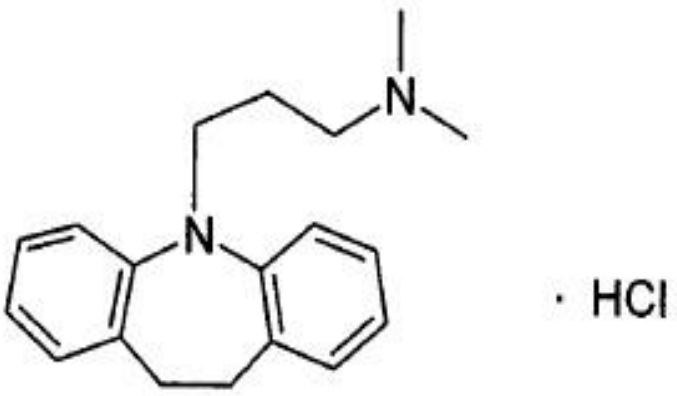


Figure 1.3: Chemical structure of amitriptyline. Reproduced from Viola *et al.* (2000) with permission, Appendix A.3.

The anti-depressive characteristic of amitriptyline is part of its chronic pain management mechanism, in addition to the peripheral activity on range of receptors and ion channels such as sodium channels, NMDA, adenosine receptors. In NP treatment guidelines, amitriptyline is considered as the first line treatment for NP like diabetic neuropathy and post-herpetic neuralgia (Breivik *et al.*, 2006; McDermott *et al.*, 2006; Micó *et al.*, 2006; NICE, 2013)

Pharmacokinetics

Amitriptyline bioavailability is between 31 to 61 % after fast, but incomplete absorption from gastrointestinal tract. The active metabolite of amitriptyline is nortriptyline. Amitriptyline is characterized by high lipid solubility and protein binding affinity (82-96%), which is vital in increasing tissue distribution. The plasma therapeutic concentration of amitriptyline is 80-200 ng/ml in doses of 75 mg to 100 mg taken orally (Waller and Renwick, 1994a).

Mechanism of action

Amitriptyline as a tricyclic antidepressant inhibits reuptake of the serotonin and noradrenaline by blocking monoamine transporter there by increasing both the amount of amines (serotonin and noradrenaline) in the intrasynaptic space and the time these amines are present in the presynaptic space. All of these actions improve serotonin transmission through post synaptic space (Richard *et al.*, 2008).

The analgesic effect of amitriptyline in NP largely mirrors its mechanism of action in depression in addition to its peripheral action of blocking a series of pain receptors (adenosine receptors, glutamate receptors) and ion channels (sodium channels, potassium channel). The analgesic capability of amitriptyline as systemic medication, has also been suggested to be mediated through the descending inhibitory pathway in which serotonin and noradrenaline are considered as endogenous analgesics (Micó *et al.*, 2006; Marks *et al.*, 2009).

Clinical pharmacology

The main indication for systemic amitriptyline is a major depression episode, treated with a daily dose between 75 and 200 mg with a maximum dose of 300 mg. In the case of NP, the starting dose is 10 mg at night up to 75 mg with maximum range 25-150 mg daily (McQuay *et al.*, 1993; NHS, 2015a). PNP such as diabetic neuropathy and post herpetic neuralgia are amongst those PNPs managed by amitriptyline (Waller and Renwick, 1994a; NICE, 2013).

Several clinical studies have applied amitriptyline topically in a range of concentrations to relieve various types of PNP conditions (Lynch *et al.*, 2005b; Kopsky and Keppel Hesselink, 2012; Mercadante, 2015). There are different analgesic mechanisms suggested for the topical amitriptyline including the blocking of ion-gated channels such as sodium and calcium channels. Amitriptyline also blocks NMDA receptors and affects adenosine receptors helping in developing analgesic action (Sawynok *et al.*, 1999; Cummins, 2009; Sawynok, 2009). The reported doses applied topically in orofacial pain range from 2% (20 mg/ml) to 10 % (100 mg/ml) using various methods to help retain the agent near the target tissue such as orabase paste or pluronic lecithin organogel with or without a stent to help prevent the “washing away” of the medicament (Padilla *et al.*, 2000; Lynch *et al.*, 2005a; Heir *et al.*, 2008; Kopsky and Keppel Hesselink, 2012; Scheinfeld, 2014).

Side effects

When using amitriptyline as a systemic medication for NP, vigilance is required as NP patients are often likely to be older and may therefore have multiple comorbidities (Gore *et al.*, 2006). This is not always the case with topical treatment of NOP, but a similar degree of vigilance is required given the medication is used “off-label”.

According to the General Medical Council (GMC), there are several aspects should be followed in case of the need for off-label prescription: the sufficient knowledge of safety and efficacy of the prescribed drugs, the medical documentation explaining the need for such prescription and the necessary follow up of the patient (GMC, 2015).

Amitriptyline's antagonism of histamine H1, muscarinic and α-adrenergic receptors, causes a group of side effects such as sleepiness, tremor, blurred vision, orthostatic hypotension, arrhythmia, withdrawal syndrome, seizure, weight gain and sexual disturbance (Waller and Renwick, 1994a; Richard *et al.*, 2008; O'Donnell and Shelton, 2011). These side effects can significantly affect patient adherence to treatment especially in a more elderly patient group where side effects can be more pronounced. Topical treatment as alternative route of administration has not been associated with major side effects except those related to a minimal systemic absorption of topical treatment causing mild symptoms such drowsiness, but this study had methodological issues (Kopsky and Keppel Hesselink, 2012).

1.5.2 Carbamazepine

Carbamazepine was first approved by the FDA in 1986 as initial or adjunctive treatment for epilepsy. Carbamazepine's chemical structure and characteristics are very similar to that of antiepileptic phenytoin (Figure 1.4), (McMillin *et al.*, 2010). Since its approval, carbamazepine has been established as effective treatment of epilepsy, bipolar syndrome, and as the drug of choice in trigeminal neuralgia (Porter and Meldrum, 2007; Leduc, 2008).

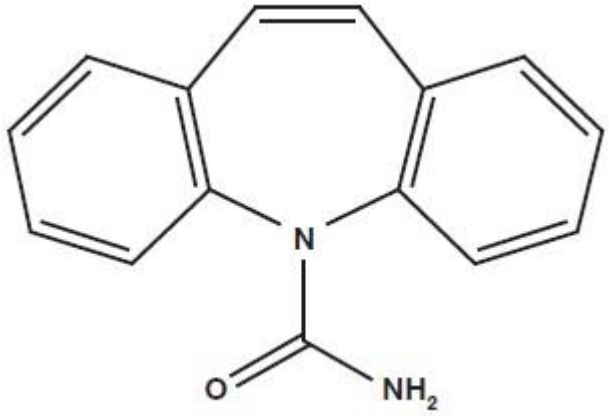


Figure 1.4: Chemical Structure of carbamazepine. Reproduced from McMillin *et al.* (2010) with permission, Appendix A.4.

Mechanism of action

The main anti-convulsant action of carbamazepine is related to blocking the voltage gated sodium channel and preventing the spread of the action potentials of a seizure. Some evidence also suggests that carbamazepine exerts an antagonistic effect against adenosine receptors and NMDA receptors (Hough *et al.*, 1996; Porter and Meldrum, 2007; Lee and Abrahams, 2008).

Pharmacokinetics

Carbamazepine is slowly, but completely, absorbed after oral administration. Carbamazepine's peak plasma concentration depends mainly on tablet type. In the extended release tablet it takes 3-12 hours while in the in the immediate release tablet it attains peak plasma concentration after 4-5 hours. Carbamazepine is distributed with 70 % bound to plasma protein with therapeutic plasma concentrations of 4-12 μg/ml (Porter and Meldrum, 2007; Leduc, 2008).

Clinical pharmacology

Carbamazepine is approved for use in both partial and generalized seizure disorders. In case of NP such as trigeminal neuralgia and diabetic neuropathy, the starting dose is 100 mg 1-2/ daily, then changed according to response up to 1.6 g/ day, the usual dose is 200 mg 3-4/day (NHS, 2015b).

Carbamazepine's sodium channel blocking action, alongside a possible antagonism on adenosine and NMDA receptors has resulted in it being applied as an adjunctive analgesic in compounded topical treatment (Vučković *et al.*, 2006; Cummins, 2009). The inclusion of carbamazepine in topical treatment produced promising results when trials were conducted to treat NOP with a 4 % (40 mg/ml) prescribed dose (Padilla *et al.*, 2000; Heir *et al.*, 2008).

Side effects

No reported side effects were found in the literature following topical treatment prescription. All the side effects mentioned in the literature related to systemic treatment.

Diplopia and ataxia are the main side effects which can be managed by dose adjustment. In case of higher doses drowsiness, hyponatremia and water intoxication may occur. In the case of prolonged systemic treatment for example in trigeminal neuralgia patients, idiosyncratic blood dyscrasias including fatal cases of aplastic anaemia have been reported in the first four months of treatment. The most common idiosyncratic reaction is erythematous skin rush. Mild persistent leukopenia has also been reported in some patients which needs careful monitoring and or cessation of the drug (Porter and Meldrum, 2007; Leduc, 2008; Lee and Abrahams, 2008).

Carbamazepine has also been found to trigger Steven Johnson’s syndrome, a life threating reaction that may be related to the immune reaction to one of its metabolite (Porter and Meldrum, 2007; Leduc, 2008).

1.5.3 Gabapentin

Gabapentin was designed with a chemical structure similar to the Gama Amino-butyric acid structure allowing it to easily penetrating the blood brain barriers Figure 1.5), (Rose and Kam, 2002). Gabapentin is a water soluble amino acid used mainly in the management of epilepsy and more recently in chronic pain management especially in postherpetic neuralgia (Porter and Meldrum, 2007; Leduc, 2008; NICE, 2013).

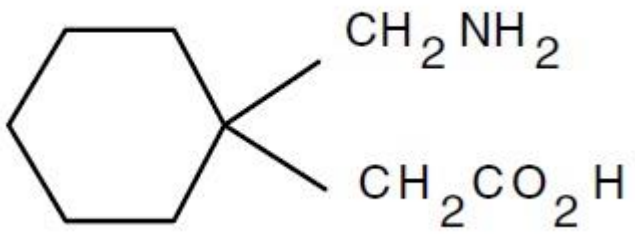


Figure 1.5: Chemical structure of gabapentin. Reproduced from Rose and Kam (2002) with permission, Appendix A.5.

Pharmacokinetics

High plasma levels of gabapentin are reached after 2-3 hours of oral administration of gabapentin with bioavailability of 60% achieved after 300 mg tablet ingestion. Gabapentin's bioavailability is subject however to L-amino acid transporter saturation and may therefore significantly decrease with higher oral doses (Porter and Meldrum, 2007).

Mechanism of action

Gabapentin's main mechanism of action is through voltage gated calcium channels by binding to $\alpha 2\delta$-subunit and blocking calcium ion influx. This decreases action potentials related to excitatory stimuli thereby decreasing the potential for seizures. Its therapeutic efficacy may also be amplified by decreasing glutamate release from presynaptic reservoir and increasing the

plasma level of gamma amino butyric acid (Porter and Meldrum, 2007; Taylor, 2009). The $\alpha 2\delta$-subunit ligand mechanism also justifies the analgesic effect of gabapentin both peripherally and centrally in management of NP.

Clinical pharmacology

Gabapentin is prescribed in case of partial and generalized seizure with daily dose up to 4800 mg in three divided doses. NICE guidelines put gabapentin in the first line of NP management especially postherpetic neuralgia. Adult dose started with 300 mg there times daily and increased according to response up to 3.6 g/day (Porter and Meldrum, 2007; Leduc, 2008; NICE, 2013; NHS, 2015c).

The topical gabapentin dose of 4 % (40 mg/ml) was found helpful in treatment of NOP. The pathway of topical analgesic is ligand to the ability of gabapentin to block the Ca $\alpha 2\delta$-1 subunit, in addition to gabapentin's role in blocking excited synapse formation (synaptogenesis) that was found associated with NOP pathophysiology (Luo *et al.*, 2002; Gordh *et al.*, 2008; Bauer *et al.*, 2009; Eroglu *et al.*, 2009; Li *et al.*, 2014; Hiom *et al.*, 2015).

Side effects

Gabapentin is generally considered a safe treatment for chronic pain conditions with side effects mainly related to dizziness, peripheral oedema and gait impairment all of which that can be controlled by dose titration. The topical use of gabapentin was not associated with any reported side effects or toxicity when applied intra or extra orally in the literature (Heir *et al.*, 2008; Hiom *et al.*, 2015).

1.6 Peripheral receptors

The biological part of pain is related to the neuron in both peripheral and central nervous system. The nociceptor part of the neuron body in PNP, expresses a group of receptors and ion channels that found essential for both pain and analgesic process (Basbaum and Woolf, 1999). The increased knowledge of molecular and signalling mechanism of pain transduction at nociceptors side, helped in raise new targets and methods of pain management including NOP. Peripheral pathophysiology of NOP as in deafferentation pain and post herpetic neuralgia suggests that there is likely to be a role for neurotransmitter receptors and excitability of ion channels in starting pain experience. The previous assuming was what the efficacy and specificity of these topical agents depend on in peripheral tissue to perform analgesic action (Henry and Hargreaves, 2007; McDougall, 2011).

The analgesic action of the three neuromodulators in this study was justified but not completely confirmed regarding expression of their main targets on nerve endings (nociceptors) in the skin or oral mucosa where the drug action and potential damage might be. In the literature, there are a group of expected targets for these NMs as peripheral analgesics will be discussed. The receptors and ion channels mentioned here are not the final and/or the exclusive pathway of peripheral analgesics but thought as the main pathway of pain analgesia:

1.6.1 Adenosine receptors

Adenosine is an endogenous nucleoside molecule with wide physiological functions. Adenosine has a neuromodulator function through four extracellular receptors: A1, A2A, A2B and A3 (Figure 1.6), (Landolt *et al.*, 2012). Their main action is through G-protein and cyclic adenosine 5-monophosphate cAMP (Doak and Sawynok, 1995). In addition to neurophysiology, these metabotropic receptors are distributed in various organs exerting numerous physiological roles in cardiac, CNS and immune system function (Jacobson, 2002).

The adenosine endogenous system has a role in anti-nociception through blocking of calcium channel excitation and inhibition of trans-synaptic transmission (Dolphin *et al.*, 1986). The same endogenous system is thought to

play the pathway of amitriptyline's analgesic mechanism, mainly by decreasing uptake of peripheral adenosine (Sawynok *et al.*, 1999).

Neuronal culture and immunohistochemical studies have reported the expression of A1 and A2A receptors in the sensory neurons of PNS (Sawynok, 2009). The anti–nociception resulting from stimulating A1 receptors leads to a decrease in cAMP production, the release of calcitonin gene related peptide and the influx of calcium ions, all of which contribute to the analgesic effects of A1 agonists. Hyperalgesia resulting from agonist stimulation of A2A receptors, results from an increase in cAMP production resulting in the synthesis of protein kinase-A, phosphorylation of sodium channels and increased in ward Na^+ current (Burnstock and Sawynok, 2010). The A2B and A3 receptors may affect pain nociception indirectly, through the stimulation of immune and inflammatory cells such as mast cells where A3 receptors are expressed thereby influencing the release of inflammatory mediators (Carruthers *et al.*, 2001; Fredholm *et al.*, 2001; Gessi *et al.*, 2008; Sawynok, 2009).

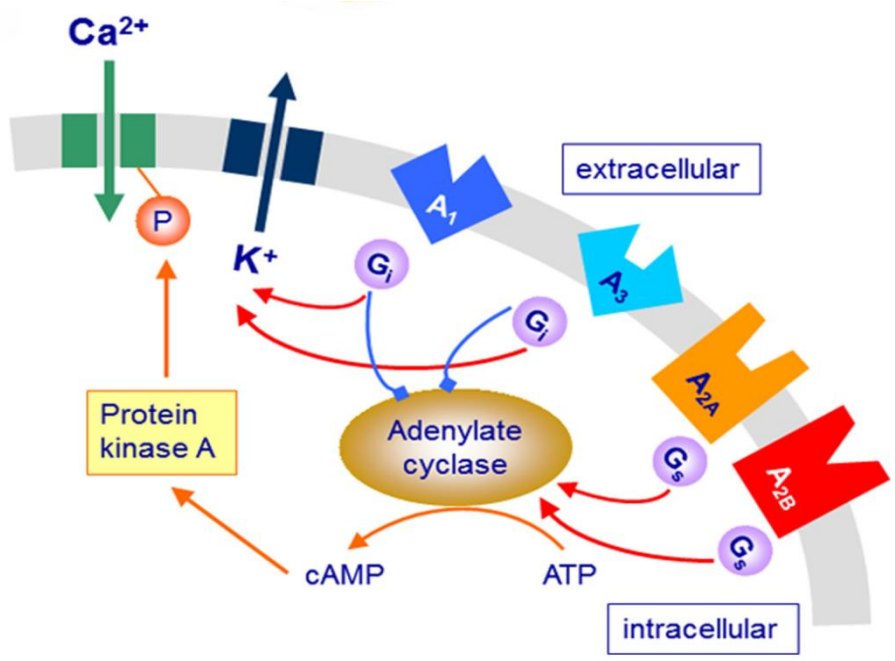


Figure 1.6: Adenosine receptors. Reproduced from Landolt *et al.* (2012), this is an open-access article.

1.6.2 Sodium channels

Sodium channels are the main voltage gated ion channels involved in pain transduction and propagation through the nervous system. The sodium channel's structure is a trans-membrane domain with a central ion-conducting pore (Figure 1.7), (Catterall, 2000). Sodium channels are composed of two main subunits: α and β subunits, the α-subunit is integral membrane protein, it represents the main functional part of sodium channel. The β-subunits are a transmembrane protein and act as a controller and a stabilizer of the α-subunit (Cummins, 2009). Sodium channels are subdivided according to their sensitivity to tetrodotoxin (TTX) into sensitive and resistant sodium channels (Lee *et al.*, 2005). The pain sensation starts peripherally when a noxious stimulus stimulates depolarization of the cell membrane and causes the sodium channels to open causing the start of the action potential and its propagation. These processes last milliseconds before the cell membrane is repolarized through the closure of the sodium channel and concomitant opening of potassium channels to allow for outflow current for repolarization (Devor, 2006; Cummins and Rush, 2007; Cummins, 2009).

The mechanism of NP is associated with various physiological changes including the excitability and distribution of sodium channels. Among these changes, there are evidence of mutation and upregulation of a group of sodium channels on myelinated and unmyelinated peripheral nociceptors in diabetic neuropathy (Faber *et al.*, 2012a; Faber *et al.*, 2012b).

Types of Sodium channels

According to the genomic sequence of a α-subunit, nine types of the sodium channel were identified: Na_v 1.1 to Na_v 1.9. The variants Na_v 1.1 to Na_v 1.6 are distributed in the nervous system, skeletal system and in the heart muscle without strong evidence about their role in pain processing or in the mechanism of analgesia except Nav 1.3, as opposed to Na_v1.7 to Na_v1.9 which are the best target for analgesia (Planells-Cases *et al.*, 2000; Yu *et al.*, 2006; Cummins, 2009).

Na_v 1.3

There is some controversy regarding the Na_v 1.3 TTX sensitive channel's role in pain nociception and analgesia. The expression of Na_v 1.3 normally subsides after nerve maturation, however, animal studies suggest that there is over expression of Na_v 1.3 TTX sensitive channels in the nerve injuries and NP role and that they play a role in ectopic pain firing and pain behaviour in neuropathic pain pathophysiology (Rogers *et al.*, 2006).

Na_v 1.7

These types of sodium channels represent the main channels involved in pain transduction and represent the optimum target for peripherally acting analgesics. Na_v 1.7 channels are TTX-sensitive channels and are exclusively only present peripherally. They are expressed in the nociceptive nerve endings of mainly C-fibres with less expression on Aδ fibres. They play a major role in normal, inflammatory and NP transduction; the latter is particularly true in diabetic neuropathy. The mutation of Na_v 1.7 channels found in recent studies of diabetic patients with PNP as a major factor in NP pathophysiology (Cummins, 2009; Faber *et al.*, 2012a).

Na_v 1.8

Na_v 1.8 TTX-resistant channels are mainly implicated in the normal pain perception in the peripheral nerve endings with controversy regarding their role in neuropathic pain pathophysiology and chronicity. It is believed that the type of nerve injury is crucial in determining the under-, or over-, expression of Na_v 1.8 channels. Mutation, redistribution on uninjured nerve with up regulation of immune-reactivity may be illustrating the Na_v 1.8 channel's role in NP pathophysiology (Gold *et al.*, 2003; Faber *et al.*, 2012b).

Na_v 1.9

Na_v 1.9 is a TTX-resistant sodium channel mainly expressed in dorsal root ganglia, but it is also expressed on C-fibres and middle and large myelinated fibres. Na_v 1.9 has a significant role in peripheral nociceptive and hyperalgesia associated with inflammatory pain. Its role in NP is less obvious given it is under

expressed following nerve injury and NP pathophysiology (Klugbauer *et al.*, 1995; Fang *et al.*, 2002; Djouhri *et al.*, 2003; Cummins, 2009).

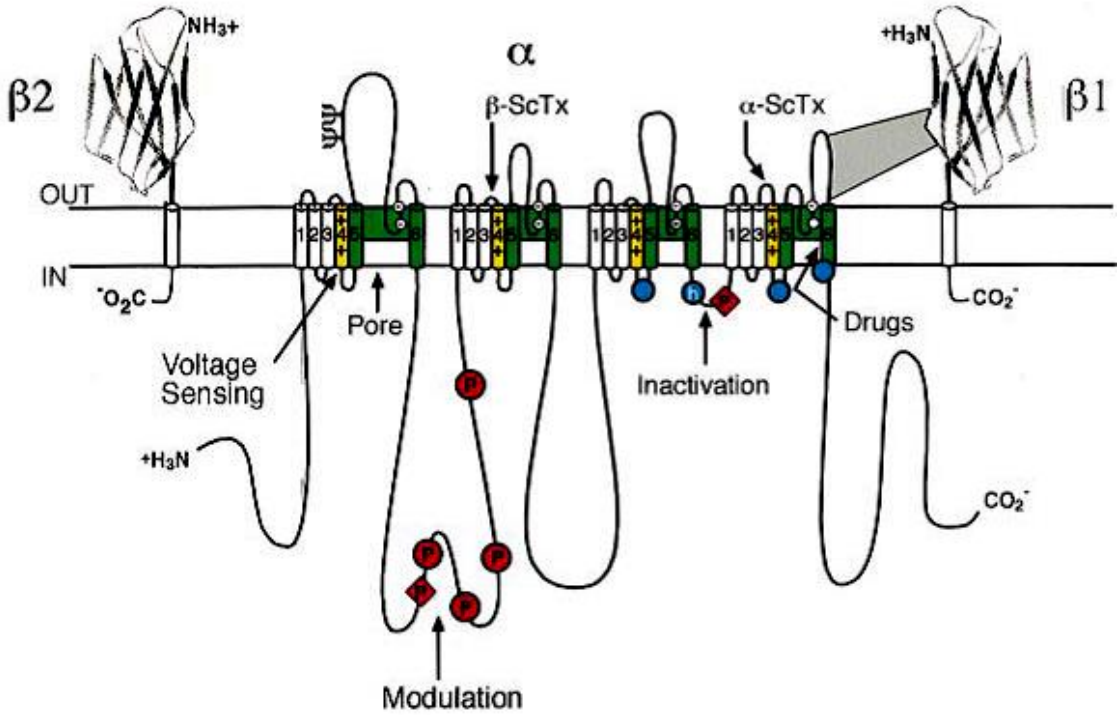


Figure 1.7: Structure of sodium channels. Reproduced from Catterall (2000) with permission, Appendix A.6.

1.6.3 Calcium channels

Calcium channels are a group of voltage-gated ion channels expressed in various tissue cells and implicated in different physiological functions. In neuronal cells, the primary function of calcium channels is to promote and propagate depolarization. Calcium channels are activated through either the binding of chemical substances or by membrane depolarization propagation (McGivern, 2009).

Calcium channels consist mainly of transmembrane pore α1-subunits, and they are the main route for calcium ion influx through the plasma membrane (Figure 1.8), (Walker and De Waard, 1998). The transmembrane pore α1-subunits contain receptors for both the activation and blocking of their pore by means of pharmacological agents. Other coalescing subunits within the calcium channel complex are the $\alpha 2\delta$ membrane ligand subunit, β cytoplasmic subunit and γ transmembrane subunit. These subunits usually modulate the kinetic of voltage gating α1 subunit in addition to membrane insertion and voltage dependence. Usually α1 subunit unites with one or two of the aforementioned subunits in channel formation.

Calcium channels are divided into high voltage activated and low voltage activated according to their activating voltage. The high voltage calcium channels include L, N, P/Q and R types and the low voltage activated are mainly T type (Ertel *et al.*, 2000; Catterall *et al.*, 2003; McGivern, 2006). The main calcium channels associated with nociception and thereby representing therapeutic targets for peripherally acting analgesia are the N, T types and auxiliary subunits (α2δ subunits), (McGivern and McDonough, 2004; McGivern, 2006; McGivern, 2009).

N-channels

N-channels are mostly found expressed on presynaptic nerve terminals in peripheral and central nervous system. N-channels are expressed as a main α1-subunit with α2δ and β subunits. Animal studies using nerve injury, ligation, or experimentally induced inflammatory states have shown that upregulation of

N-channels occurs with the chronic pain resulting from the experimentally induced injury. N-channel blocking agents such as Ziconotide have produced promising results in chronic pain management such as cancer pain (Matthews and Dickenson, 2001; Saegusa *et al.*, 2001; McGivern, 2009).

T-channels

T-channels are mostly situated centrally in the brain and spinal cord with some present peripherally in neuronal cell bodies and associated dendrites and have an excitatory role. T-channels are blocked by varying medications including anaesthetic and anti-psychotic drugs but there is still no potent and highly selective blocker available for them (Scroggs and Fox, 1992; McGivern, 2009).

Auxiliary subunits ligands

There is a wealth of evidence, mainly from animal studies, demonstrating the important role of the calcium channel subunit α2δ-1 in the analgesic pathway of anti convulsants medications (gabapentin and pregabalin). It is perhaps, therefore, unsurprising that α2δ ligands are named as a drug of choice in PNP (Maneuf *et al.*, 2006; McGivern, 2009; Dolphin, 2013). Over expression of the $\alpha 2\delta$-1 subunit at peripheral nerve endings is associated with an increased incidence of NP. Further pharmacological evidence of the importance of the $\alpha 2\delta$-1 subunit's role in maintaining and propagating PNP is found in the action of gabapentin which through binding to the $\alpha 2\delta$-1 subunit in the presynaptic neuron, inhibits neurotransmitter release and the influx of calcium ion currents thereby reducing peripheral nociceptive impulses (Stefani *et al.*, 1998; Newton *et al.*, 2001; Maneuf *et al.*, 2006; Taylor, 2009).

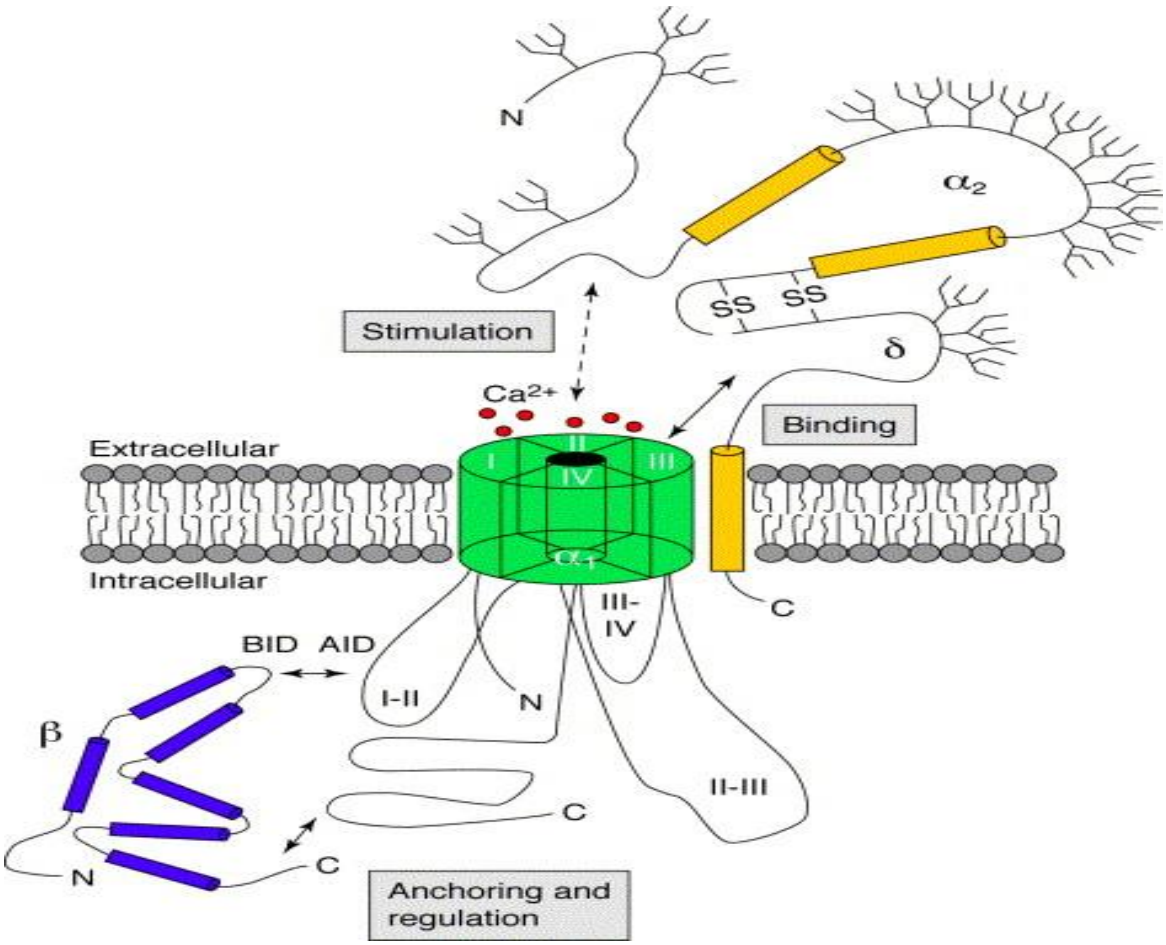


Figure 1.8: Structure of calcium channel. Reproduced from Walker and De Waard (1998) with permission, Appendix A.7.

1.6.4 NMDA receptors

The N-methyl D-aspartate (NMDA) receptors are one of the ionotropic neurotransmitter receptors, where glutamate, a principal neuro-excitatory amino acid, binds to exert its excitatory effect both in the central and peripheral nervous system. Glutamate is released peripherally following tissue injury and inflammation, and is therefore identified both in acute and chronic pain states as one contributory factor to maintenance of nervous system excitation.

When glutamate is released peripherally from afferent nerve endings it stimulates various inflammatory and pain proactive receptors like NMDA (Miller *et al.*, 2011). NMDA receptor is multi-ionic channel that is permeable for sodium, calcium and potassium ions following glutamate depolarization stage. The channel consists of three types of subunits: NMDA1, NMDA2 (A, B, C, D) and NMDA3 (A, B), (Figure 1.9), (Lakhan *et al.*, 2013). The channel structure is largely made up of NMDA1/NMDA2 subunits; however, NMDA3 may replace NMDA2 subunits resulting in a channel with less permeability for Ca^{++}. In peripheral tissue, the dominate expression of NMDA receptor is owned mainly by NMDA1/NMDA2B and NMDA1/NMDA2D subunits.

The expression of NMDA receptors in the dorsal root ganglia and trigeminal ganglia along with peripheral expression has been reported in animal studies (Coggeshall and Carlton, 1998; Li *et al.*, 2004). Topical treatment with drugs possessing NMDA-antagonist properties such as amitriptyline, carbamazepine and ketamine has been found helpful in relieving nociceptive impulses and NP. (Mathisen *et al.*, 1995; Lynch *et al.*, 2005a; Cairns, 2009a).

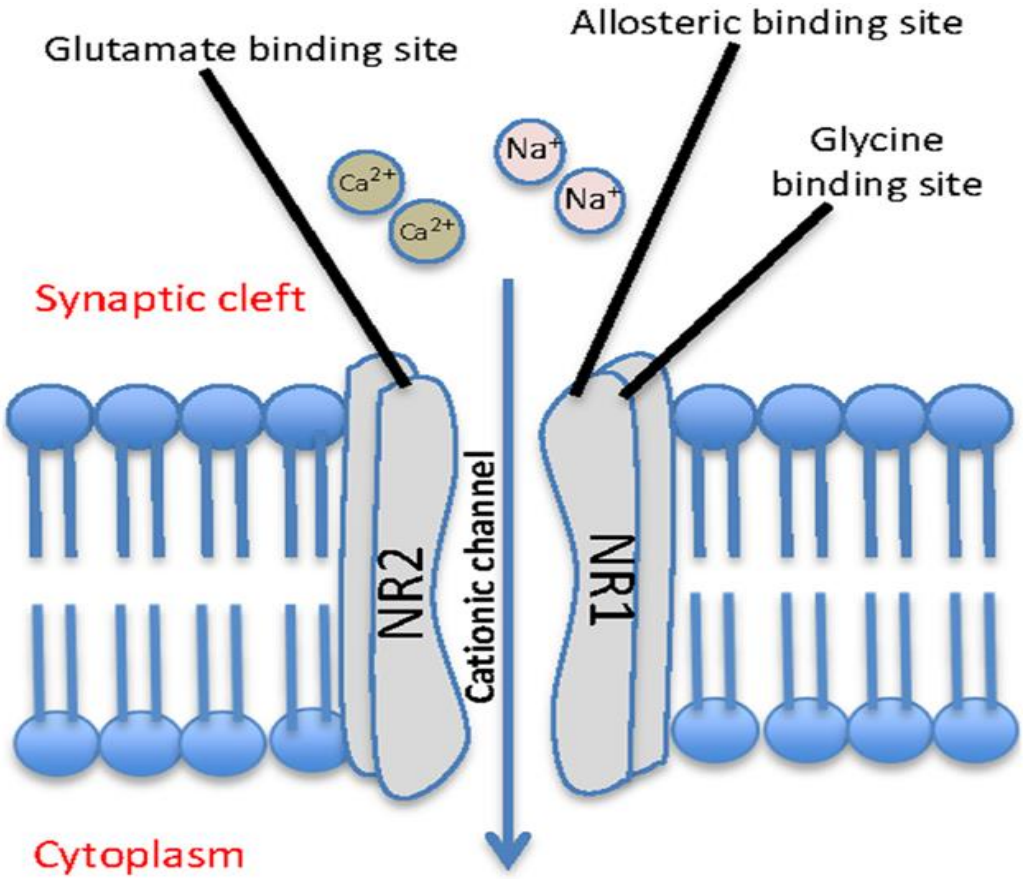


Figure 1.9: NMDA receptor. Reproduced from Lakhan *et al.* (2013), this an open-access article.

1.7 Compounded drugs and Drug approval process

Patient satisfaction and wellbeing is the primary goal of clinicians and their treatment plans. Compounded drugs are a good example of clinicians trying to achieve this aim through treatment modification to alleviate patient's suffering and increase their satisfaction by decreasing side effects. The United State food and drug administration (FDA) defined compounding as "*a practice in which a licensed pharmacist, a licensed physician, or, in the case of an outsourcing facility, a person under the supervision of a licensed pharmacist, combines, mixes, or alters ingredients of a drug to create a medication tailored to the needs of an individual patient*" (FDA, 2015a). Recently, concerns were raised regarding the potency and purity of compounded medications with increased marketing of these pharmaceutical products with less control and monitoring especially in Europe (FDA, 2006; Gudeman *et al.*, 2013; Minghetti *et al.*, 2014; Phillips *et al.*, 2015).

The treatment of NOP can be frustrating for the patient and clinician alike as finding agents to alleviate the suffering can be benefits from the patient's point of view (Durham and Nixdorf, 2014). It is perhaps this challenge that has driven the desire to compound topical formulations of agents known to be effective systemically for NOP. The issue of topical treatment toxicity is less well addressed and reported as there are only low power and uncontrolled clinical studies examining the topical application of neuromodulatory medications in NOP.

The programme of research in this thesis came about due to the concerns of the research team about the safety of compounded topical treatment, because of the lack of evidence that this administration route had been sufficiently examine to be approved for use. The standard drug approval process comprises long and expensive steps of drug design, efficacy and safety procedures, and can result in a high percentage of drugs being rejected after the process is complete because of safety or toxicity problems (Hopkins, 2008).

The drug approval process contains three main steps: Discovery and design, preclinical and clinical stage, in addition to the post authorization monitoring

(FDA, 2015b). The cytotoxicity of a new drug is tested by what is defined as the pre-clinical investigations and guidelines, these investigations can be simply summarised as: Single and repeated dose safety, genotoxicity, carcinogenicity, reproductive and developmental toxicity, photo-toxicity, hepatotoxicity, mitochondrial toxicity and drug dependence (EMA, 2015b; FDA, 2015c).

The European Medicines Agency (EMA), recently included new guidelines in the preclinical drug approval process, which is the local tolerance of the drug. The new guidelines states that the drug cytotoxicity should be tested *in vitro* on tissues that will intentionally or accidentally be in direct contact with the drug. Unfortunately, the new guidelines will start in May 2016 and do not include drugs now used globally as compounded topical treatment for patient suffering chronic sickness such as NP (EMA, 2015a).

1.8 Cell lines and tissues

Prior to any human clinical trials, *in vitro* cellular investigation, with all its limitations and advantages, is the classical method for preclinical testing and prediction of the expected toxicity. The selection of *in vitro* monolayer cell line models and methods of investigations are expected to be representative of both the target tissue and clinical course of the drug when used clinically (Astashkina *et al.*, 2012).

Monolayer cell line testing has the benefit of reproducibility and availability especially if multiple tests are to be conducted at different times. The reproducibility of results is not otherwise always guaranteed if primary cell culture is used instead as the investigation model. The primary cell culture is a logical *in vitro* representative model, but its limitations have resulted in less use in *in vitro* studies. Among these limitations are: untoward biological effect of *in vitro* environment itself as opposed to the drug under test; limitation of patient and donor ethical requirement and passage number restriction especially when further studies to be conducted (Ekwall *et al.*, 1991; Ch, 2009).

Different human and animal cell lines were chosen in this study as representative of orofacial tissue. The selection was based on the tissue expected to be in direct prolonged, contact with topical agents. These cell lines have already been employed in different studies to investigate various orofacial reactions and cytotoxicity resonances to different materials:

1- Mouse fibroblast, 3T3 cell line was used as representative of gingival fibroblast as previously employed (Geurtsen *et al.*, 1998; Pereira *et al.*, 2002).
2- Immortalized oral keratinocytes from floor of mouth OKF6-TERT1 cell line was selected as representative of oral keratinocytes having already been used in the literature in this respect (Dickson *et al.*, 2000; Oviir *et al.*, 2006; McLeod *et al.*, 2014).
3- Skin keratinocytes as one of the expected tissues exposed to the topical treatment if used extra-orally: the HaCat cell line was used as representative of skin keratinocytes with multiple applications in skin

reaction and investigations (Boukamp *et al.*, 1988; Moharamzadeh *et al.*, 2007).

4- Primary gingival fibroblast cells were used as representative of gingival fibroblast and implemented in the in-house 3D tissue construction. These cells were collected from biopsy after routine dental treatment following ethical approval (no. 09/ H1308/ 66) and consent (Colley *et al.*, 2011).
5- The keratinocyte compartment of the in-house constructed cells was FNB6 cell line. These normal oral keratinocytes are immortalized cells obtained from dysplastic biopsy (Muntoni *et al.*, 2003).
6- MatTek model was used in this study was bought from MatTek corporation as representative of oral gingival tissue, made by primary cells (oral keratinocytes and gingival fibroblasts) with early passages (MatTek, 2015).

1.9 Summery

The systemic treatment of NOP is usually associated with an intolerable side effect profile that affects patient's adherence to treatment. Topical management offers the chance to avoid such heavy burden of side effects and deliver pain relief in mild and moderate cases (Heir *et al.*, 2008). This may have helped in evolving and progressing topical compounded treatment. In spite of the limitations of clinical studies that have employed topical medications in the orofacial region, many studies revealed the wide acceptance and application of topical medication in management of NP all over the body including NOP (Epstein *et al.*, 1997; Padilla *et al.*, 2000; Sawynok, 2003; Strümper and Durieux, 2003; Argoff, 2004; Vidal *et al.*, 2004; Lynch *et al.*, 2005b; de Leon-Casasola, 2007; Heir *et al.*, 2008; McCleane, 2010; Jorge *et al.*, 2011; Kopsky and Keppel Hesselink, 2012; Haribabu *et al.*, 2013; Sawynok, 2014; Thompson and Brooks, 2015). The NMs employed in topical treatment have the approval for systemic administration, but have only been designed for this administration (FDA, 2015a). Very few studies have tested the topical effects of NMs on human cells. Some have suggested that there is evidence of cell death and limitation of cellular proliferation (Yun *et al.*, 2005; Moreno-Fernández *et al.*, 2008) following topical application. This, therefore, raises important questions about the potential untoward local effects caused by these drugs when applied to the orofacial tissue which tends to have a high cellular turnover rate. This also becomes especially pertinent if topical administration of the NMs to the oral mucosa and facial skin proves effective in the short-term and is then prescribed as a regular long-term medication. In this study we will investigate the effects of selected topical drugs on cell lines and tissue relevant to oral tissue *in vitro*.

1.10 Aims and Objectives

The aim of this study was to investigate the effect of pharmacological concentrations of three neuromodulatory medications (amitriptyline, carbamazepine and gabapentin) frequently reported to be applied topically on cell lines and tissue relevant to the orofacial tissue *in vitro*.

The specific objectives were:

Phase 1 Using monolayer cell culture to investigate:

1- Both cumulative and specific time cellular viability using alamarBlue® assay against a range of concentrations of the three NMs.
2- Cellular counting using CCK-8 assay against a range of concentrations of the three NMs using specific time exposure.
3- Cellular morphology and attachment following 30 min exposure to the HPLC concentrations of the three NMs using scanning electron microscope.
4- The expression of 20 different Cytokines after 30 min exposure to the HPLC concentrations of the three NMs.

Phase 2 Using 3D models to investigate:

1- The effect of amitriptyline on 3D tissue viability and cytotoxicity at different time points using alamarBlue® and LDH assays.
2- Amitriptyline transport through the 3D tissue using anti-amitriptyline antibody in immunofluorescence technique and HPLC.
3- The effect of amitriptyline on 3D tissue morphology and apoptosis activity using H&E and anti-caspase 3 antibody respectively.

Phase 3 Using RNA from total human gingival tissue biopsy and SH-SY5Y cell line to identify the expression of receptors to the three NMs.

Chapter: 2 Materials and Methods

2.1 Tissue culture techniques

All media and supplements were obtained from Sigma Aldrich, UK unless otherwise stated. Tissue culture procedures were carried out in Class II laminar hood (BioAir SafeFlow 1.2, Lafteck, Australia) in the tissue culture laboratory using aseptic techniques. An Incubator was used to maintain the tissue flask at 37 °C, 95 % O_2 and 5 % CO_2 (Incu-Safe, Sanyo, Japan). 3T3 and HaCat cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC), OKF6-TERT1 cell line was a gift from Dr. Max Robinson, Newcastle University.

2.1.1 Growth and maintenance of 3T3 mouse fibroblast

Mouse fibroblasts 3T3 cells passage 5, were propagated in Dulbecco-Modified Eagles Media (DMEM) with glutamine, 10 % (v/v) foetal bovine serum and 1 % (v/v, 100 U/100 µg) of penicillin /streptomycin. Mouse 3T3 cells were seeded in 25 cm^2 (T 25) flasks with 5ml of culture medium. The growth medium was replaced every day with fresh culture medium. Cells were passaged at ratio of 1:5 every 3-4 days or when 80 % confluency was reached, which was assessed by light microscopy (Leica, UK). Cells were washed with phosphate buffered saline (PBS: 137 mM $NaCl_2$, 2.7 mM KCl, 4.3 mM. $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.3) after discarding old growth medium. Then 3 ml of 0.05 % trypsin/ 0.2 % Ethylenediaminetetraacetic acid (v/v, EDTA) was added and the flask incubated for 3-5 min, to insure cell detachment. After cell detachment, the trypsin was neutralized with fresh 7 ml culture medium. Cells were seeded at approximately 1 million cells / flask into 75 cm^2 flask (T 75).

2.1.2 Growth and maintenance of human oral keratinocyte cells (OKF6-TERT1)

The Keratinocyte-Serum Free Media with glutamine (K-SFM), bovine pituitary extract and epidermal growth factor were obtained from Life Technologies, UK. The growth media was prepared by adding bovine pituitary extract (30 µg/ml), epidermal growth factor (0.1 ng/ml), calcium chloride (0.3 mM) and 5 ml

penicillin /streptomycin to K-SFM. A frozen stock of OKF6-TERT1 cells at passage 3 were initially seeded in 25 cm^2 flask (T 25) with 5 ml culture medium. Cells were passaged 7-9 days later or when 80 % confluency was reached. Cells were trypsinized with 3 ml of trypsin/EDTA added to the flask for 30 second after discarding the media, then a new 3 ml trypsin used and the flask kept in the incubator for 8-10 min. When the cells detached, 15 ml of DMEM was used to neutralize the trypsin, and it was centrifuged at 2000 rpm for 10 min. Cells were then seeded in new flask at density of 1 million / flask with fresh K-SFM media.

2.1.3 Growth and maintenance of human skin keratinocytes cells (HaCat)

HaCat cells at passage 70 were propagated in the DMEM media (Section 2.1.1) and passaged after 4-5 days or after 80 % confluency was reached. After aspirating the culture media, the flask washed twice with EDTA, then 3 ml trypsin/EDTA added and the flask incubated for 5-10 min. A fresh media was added and the suspension centrifuged for 5 min at 1200 rpm. The media was then replaced with fresh culture media and the cells seeded as new passage in 75 cm^2 flask (T 75). Cells were seeded at approximately 1 million cells / flask into 75 cm^2 flask (T 75).

2.1.4 Growth and maintenance of primary human gingival fibroblast

Primary human gingival fibroblasts cells at passage 3 was a gift from School of Clinical Dentistry/ University of Sheffield. Cells were propagated in Dulbecco-Modified Eagles Media (DMEM) with glutamine, 10 % (v/v) foetal bovine serum and 1 % (v/v, 100 U/100 µg) of penicillin /streptomycin. Cells were passaged at ratio of 1:3 every when 80 % confluency was reached. Cells were washed with phosphate buffered saline then 3 ml of trypsin/ EDTA was added and the flask incubated for 3-5 min, to insure cell detachment. After cell detachment, the trypsin was neutralized with fresh 7 ml culture medium. Cells were seeded at approximately 1 million cells / flask into 75 cm^2 flask (T 75).

2.2 Preparation of drugs for use in cell culture

Amitriptyline hydrochloride, gabapentin and carbamazepine were purchased in pure sterilized form from Sigma-Aldrich, UK. All the preparations and dilutions were made in the Class II hood. Manufacturer instructions were followed in risk and safety measures.

2.2.1 Amitriptyline hydrochloride

Amitriptyline was initially dissolved in double–distilled water (dd H_2O) to concentration of 1 % (w/v) and stored in the dark at 4 ◦C. The working concentrations were made by further dilution in PBS first and then culture media. Concentrations are shown in Table 2.1:

Volume 0.07% drug solution (µl)	Volume of media (µl)	Concentrations (µM)
14	1486	20
34	1466	50
68	1432	100
136	1364	200
272	1228	400
409	1829	600
544	966	800
681	819	1000
818	682	1200
886	614	1300
954	540	1400
357(0.2%)	1143	1500
380(0.2%)	1120	1600
404(0.2%)	1096	1700
428(0.2%)	1072	1800

Table 2.1: Amitriptyline's range of concentrations that was used in cumulative cellular viability experiments in 3T3, HaCat and OKF6-TERT1 cell lines using alamarBlue®.

2.2.2 Gabapentin

Gabapentin was dissolved in dd H_2O to concentration of 0.5 % (w/v) solution and stored in the dark container at 4 °C. Further dilution was made (0.017 %, w/v) before the required concentrations were prepared with appropriate culture media according to the cell line type. Concentrations are shown in

Table 2.2:

Volume 0.017% drug solution (µl)	**Volume of media (µl)**	**Concentrations (µM)**
13.5	2236.5	6
67.5	2182.5	30
135	2115	60
225	2025	100
337.5	1912.5	150
450	1800	200
562.5	1687.5	250
675	1575	300
900	1350	400
1125	1125	500
1350	900	600
1575	675	700
1800	450	800
2025	225	900
380 (0.5% drug solution)	1620	5540

Table 2.2: Gabapentin's range of concentrations that was used in cumulative cellular viability experiments in 3T3, HaCat and OKF6-TERT1 cell lines using alamarBlue®.

2.2.3 Carbamazepine

Carbamazepine was initially dissolved in 100 % (v/v) ethanol to concentration of 2 % (w/v) solution and stored in the dark at room temperature. To create working concentrations, further dilution was made (0.07%, w/v) in culture media before designed concentrations were made as shown in

Table 2.3:

Volume 0.07% drug solution (µl)	**Volume of media (µl)**	**Concentrations (µM)**
5	1495	10
15	1485	30
30	1470	60
50	1450	100
75	1425	150
100	1400	200
150	1350	300
200	1300	400
250	1250	500
300	1200	600
350	1150	700
400	1100	800
500	1000	1000
550	950	1100
650	850	1300
750	750	1500
850	650	1700
1000	500	2000
1050	450	2100

Table 2.3: Carbamazepine's range of concentrations was employed in cumulative cellular viability experiments in 3T3, HaCat and OKF6-TERT1 cell lines using alamarBlue®.

2.3 Monolayer cell culture viability experiments

AlamarBlue® (AbD Serotec, UK) was used to establish cellular viability. The differences in chemical reduction of an indicator (Resazurin) were measured in the growth media reflecting cellular viability.

2.3.1 Cell density and incubation time

Firstly, appropriate cell line density and appropriate incubation time were established for each cell line. Each cell type were seeded at density of 5000, 10000 and 20000 (+ 25,000 for OKF6-TERT1 cell line) cells per well in 96 well plate and culture media was added to a final volume of 200 µl per well. After overnight incubation at 37 ◦C, the media was replaced with different concentrations of drug in addition to 10 % (v/v) alamarBlue®. Absorbance was measured at two wavelengths (570/600) nm using a plate reader (BioTek, Winooski, VT) at 30 min, 1 h, 2 h, 4 h and 24 h. The cell viability was calculated using the following equation:

$$\textbf{Percentage reduction of alamarBlue®} = \frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} * 100$$

Where:

O1 = molar extinction coefficient (E) of oxidized alamarBlue® (Blue) at 570 nm

O2= E of oxidized alamarBlue at 600 nm

R1 = E of reduced alamarBlue (Red) at 570 nm

R2= E of reduced alamarBlue at 600 nm

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

N1 = absorbance of negative control well (media plus alamarBlue® but no cells) at 570 nm

N2 = absorbance of negative control well (media plus alamarBlue but no cells) at 600 nm

2.3.2 Cumulative cell viability assay

The information for the cell density and incubation time was found in the previous experiment (Section 2.3.1). The 3T3, OKF6-TERT1 and HaCat cells were trypsinized and seeded at 10000, 20000,10000 cells per well respectively, in a 96-well plate to a final volume of 200 μl per well, being incubated overnight at 37 ◦C. Media was then replaced by medication solution at one of the tested concentrations plus 10% (v/v) of alamarBlue®. The plate reader was used to measure absorbance at (570/600) nm at 30 min, 1 h, 2 h, 4 h and 24 h time points. The percentage difference in reduction between control and treated cells was calculated using the following equation:

$$\text{Percentage difference between controls and treated cells} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} * 100$$

Where:

O1 = molar extinction coefficient (E) of oxidized alamarBlue® (Blue) at 570 nm

O2= E of oxidized alamarBlue at 600 nm

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

P1 = absorbance of positive growth control well

(cells plus alamarBlue but no test agent) at 570 nm

P2 = absorbance of positive growth control well

(cells plus alamarBlue but no test agent) at 600 nm

The results represent the cytotoxicity effect of the drugs on cells viability.

2.3.3 Effect of NMs at specific time points

The keratinocytes cells, HaCat and OKF6-TERT1, were used in similar culture and counting as in the cumulative method. The cells were seeded in final volume of 100 µl/well in 96 well plate. After drug exposure at 30 min and 24 h, cells were washed with PBS before adding fresh culture media and 10% alamarBlue®. High and low concentrations (Table 2.4) were used and the absorbance was measured 4 h after the addition of alamarBlue®, using the same wavelengths and calculation method (Section 2.3.2).

Medications	**Low concentration (µM)**	**High concentration (mM)**
Amitriptyline	200	1.8
Carbamazepine	100	1.7
Gabapentin	150	5.54

Table 2.4: High and low concentrations of NMs were used to investigate viability change in specific time points experiment in HaCat and OKF6-TERT1 cell lines using alamarBlue®.

2.3.4 Calculation of median lethal dose (LD_{50})

Two different colorimetric assays (alamarBlue®, MTT) were used to investigate the concentrations of NMs required to kill 50 % of the cultured cells.

a- AlamarBlue® assay

The LD_{50} values of NMs were investigated in the three cell lines (3T3, HaCat and OKF6-TERT1). Using both exposure methods, the cumulative and specific time, the cell culture method were carried out as in Section 2.3.2 and 2.3.3 respectively. The concentration of NMs (Table 2.1, Table 2.2, Table 2.3), were used to conduct the experiments. The calculations were made using the following equations and the results were transferred to semi log graph paper to find the LD_{50} value:

AO_{LW} = Absorbance of AB in media – Absorbance of media only

AO_{HW} = Absorbance of AB in media – Absorbance of media only

$R_0 = AO_{LW}/AO_{HW}$

$$\text{Percentage difference in reduction} = \frac{\mathbf{A_{LW} - (A_{HW} \times R_0) \text{ for test well}}}{\mathbf{ALW - (AHW \times R0) \text{ for control well}}} \times 100$$

Where:

AOLW = absorbance of oxidized form at the lower wavelength

AOHW = absorbance of oxidized form at the higher wavelength

ALW = absorbance at lower wavelength minus the media blank

AHW = absorbance at higher wavelength minus the media blank

R0 = Correction factor

b- MTT assay

MTT assay was used to investigate the LD_{50} value for amitriptyline in FNB6 cell line. The cells were trypsinized and seeded as 5000/ well for LD_{50} experiment, a range of concentrations of amitriptyline was prepared in FNB6 culture media (Table 2.1). After 24 h incubation, the old media replaced with medication solution at one of the amitriptyline concentrations. After incubation for 30 min and 24 h, the media was discarded and the wells were washed with PBS before adding new media + 10 % (v/v) of MTT assay. Following 2 h incubation, the MTT media was discarded and a solubilisation/stop Solution [10% Triton-X 100 in acidic Isopropanol (0.1N HCl)] of 100 µl/well was added for 30 second before absorbance reading using plate read at 570 nm.

2.4 Cell counting experiments in monolayer cell culture

Cell Counting kit-8 (CCK-8) from Dojindo Laboratories (Kumamoto, Japan), was used to investigate the effects of the tested three NMs on the cell counts *in vitro*. The assay mechanism is based on cellular activity, specifically dehydrogenases and mitochondrial activity, resulting in reduction of Tetrazolium salts (yellow) to Formazan (orange) that can be quantified by plate reader in growth media.

HaCat and OKF6-TERT1 cells were seeded and treated as in Section 2.3.3, alongside a range of control cells seeded at different cell densities to establish a calibration curve. Calibration curves were made by seeding the cells at different mounting count for HaCat and OKF6-TERT1 cells, Table 2.5 .

After incubation for 30 min and 24 h, the effect of the NM was stopped by washing the cells with PBS. Cells were then incubated with 100 µl fresh culture media plus 10 % (v/v) CCK-8 reagent. Absorbance was measured after incubation for 150 min using a plate reader (BioTek, Winooski, VT) at 450 nm wavelength. The results were drawn against the calibration curve to estimate the final change in cell count after NMs exposure.

HaCat cells cell/ well		**OKF6-TERT1 cells cell/well**	
Calibration curve counts	Test count	Calibration curve counts	Test count
1,000	10,000	2,000	20,000
2,000		5,000	
5,000		10,000	
10,000		15,000	
15,000		20,000	
20,000		25,000	

Table 2.5: Cell counts in calibration curve made to estimate the effect of NMs exposure on HaCat and OKF6-TERT1 cells *in vitro.*

2.5 3D tissue cell culture

Two types of 3D tissues were used in the amitriptyline investigation: a cell line model (in-house constructed) and a primary cell model (MatTek). Culture media used in tissue maintenance depended upon cell type used to construct the 3D model, but mainly consisted of DMEM: F-12HAM, with group of additives to induce tissue growth and differentiation such as 10 ng/ml epidermal growth factor, 0.4 µg/ml hydrocortisone and 5 µg/ml insulin.

2.5.1 In-house constructed 3D model

The 3D tissue was constructed and tested in the laboratories of School of Clinical Dentistry/ University of Sheffield. Two types of cells were implemented: FNB6 cell line, representing oral keratinocyte as the top layers (250, 000 cells / transwell plate) and the second cell type was primary human gingival fibroblast (250, 000 cells / transwell plate); both were seeded onto a rat-tail collagen in a transwell plate with 0.4 µm pore size (Corning, USA). After 21 day incubation, the tissue was implemented in the experiments. The media were replaced every day with 500 µl of culture media in the lower chamber of transwell plate.

2.5.2 MatTek 3D tissue model

Tissue models were bought from MatTek Corporation, USA, Figure 2.1. Tissue was constructed using primary gingival keratinocytes and fibroblast that seeded on collagen coated membrane with pore size 0.4 µm, inner diameter = 0.875 cm. Surface area = 0.6 cm2 using MillicellTM CM microporous membrane tissue culture inserts (Millipore Corporation, Bedford, MA). Trans-epithelial electrical resistance (TEER) of each lot is measured prior to shipment (QC specification: GIN-100: 516 + 122 ohms*cm^2).

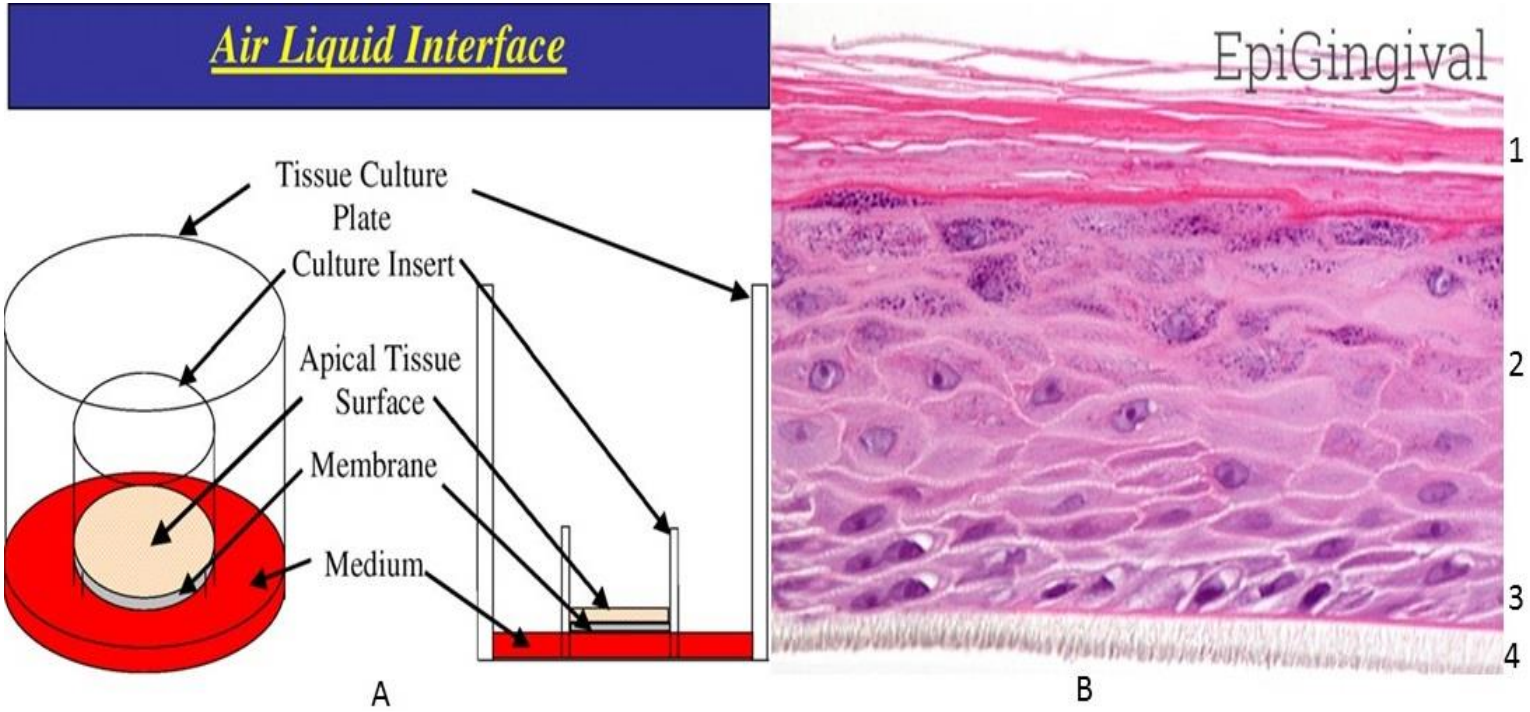


Figure 2.1: The cell culture of 3D tissue (A) air liquid interface tissue culture using tissue inserts (B) layers of full thickness gingival tissue from MatTek. 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer. Reproduced from Klausner *et al.* (2007) with permission, Appendix A.8

2.6 3D tissue viability experiments

AlamarBlue® was used to investigate viability in response to amitriptyline exposure in both 3D models. AlamarBlue® assay time was 2 h in the in-house constructed 3D model and 6 h in the MatTek 3D model. Amitriptyline concentration was 226 µM in both models and all time points.

For in-house constructed model, two time points were used 30 min and 24 h; in MatTek models, tissue was exposed to amitriptyline at the following time points: 30 min, 24 h, twice daily (B.I.D.) and three times a day (T.I.D.) for three days.

2.6.1 Viability in both 3D models at 30 min and 24 h

Three tissue inserts were used for test and control. Amitriptyline solution (226 µM) was applied in 100 µl volume on the top of the tissues and media only for the control tissues. For the lower chamber, 1 ml of fresh media was applied for both test and control tissues. Either after 30 min or 24 h incubation, the tissue inserts were transferred for a new 6 well plate and washed twice with PBS. The old media was stored, and an aliquot was sent for Amitriptyline quantification by HPLC. The tissue inserts were then incubated with 500 ml of media plus 10 % (v/v) alamarBlue® on the top in new 6-well plate. The incubation time was 6 h in MatTek models and 2 h in in-house models. The media were then aspirated and placed in 96-well plate with 100 ul/well to measure absorbance by plate reader at (570/600) nm.

2.6.2 Repeated exposure viability assay

The 3D tissue models were exposed to amitriptyline as in the 30 min exposure but instead of adding media plus alamarBlue® after 30 min, tissue inserts were replaced in a new 6 well plate with 1 ml fresh media in the lower chamber after washing, for further exposures. These procedures were repeated twice and three times daily, using separated sets of tissue, for three days before the alamarBlue® testing were done.

2.7 Lactate dehydrogenase assay (LDH), cytotoxicity assay

Culture media from viability experiments in both 3D models (The MatTek and in –house constructed) was stored at -20 °C and used in the LDH assay (CYTOTOX96®, Promega, UK). The media from 3D tissue experiments were thawed and transferred to 96-well plate as 50 µl per well (n=3) and then 50 µl/well of reconstituted substrate mix was added. After 30 min incubation at room temperature in the dark, 50 µl of stop solution was added and absorbance was measured by plate reader (BioTek, Winooski, VT) at 490 nm.

2.8 Doubling up technique to compound medications in Orabase paste

For these experiments, a sterile, without additives, Orabase paste (ConvaTec UK, Flintshire, UK), (containing: gelatin, pectin, sodium carbnoxymethyl cellulose, polythene and liquid paraffin) was used. The required amount of the pure medication and paste were weighed and placed separately on a glass tile in the Class II hood (Table 2.6). The Doubling up technique was used to mix small equal amount of powder and paste volume by spatula using the shearing technique. The process was repeated until all the powder was incorporated into the paste. The powder/paste compound was then mixed with an equal volume of fresh remaining paste and this step repeated until all the paste was used and a homogenous compound was formed.

Types (w/v)	Amount of Pure medication (mg)	Amount of Orabase paste (g)
2 % Amitriptyline	20	1
4 % Carbamazepine	40	1
4 % Gabapentin	40	1

Table 2.6: List of drugs and Orabase paste weights, were used to compound NMs medications using doubling up technique.

2.8.1 Determining drug release from the paste

A decided amount (2 g) of compounded paste of each medication were applied with a small spatula on the walls of the 6-well plate filled with 10 ml of culture media. The plate was incubated at 37 °C. After incubation for 30 min and 1 h, 2 ml media was aspirated for each time points. The media was sent to Cardiff toxicology laboratories to measure drug release from the Orabase paste by HPLC at different time points, the experiments done in replicate.

Conditions used in HPLC investigation are shown in Table 2.7.

Instrument	Perkin Elmer Flexar
Column	Spherisorb CNRP 5µ, 25cm (Column Position :1)
Column Temperature	Ambient
Flow rate	2 ml/min
Mobile Phase	Acetonitrile: 10 mM phosphate buffer,pH 3.0 (30:70)
Detector	PerkinElmer Flexar PDA Analytical Wavelength : 210 nm

Table 2.7: The conditions of HPLC used by Cardiff toxicology laboratories to investigate NMs concentrations in the culture media during drug release experiments and in the amitriptyline passage experiments in the 3D tissue cell culture.

2.9 Scanning electron microscopy

Cells were seeded onto glass cover slips at density of 100,000 per well for 3T3 cells and HaCat cells; and 200,000 per well for OKF6-TERT1 cells. After overnight incubation, media was replaced with media containing drug concentrations at a level dictated by the HPLC results. After 30 min incubation, the spent media were stored at -20 °C freezer for use with Human Inflammation Antibody Array experiment. The wells were then washed with PBS and left at 4 ˚C fridge with 2 % (v/v) Glutaraldehyde in Sorenson's phosphate buffer for fixation. Twenty four hours later, this was replaced by the following dehydration steps: 25 % ethanol for 30 min, 50 % ethanol for 30 min, 75 % ethanol for 30 min and finally the cover slips were immersed with 100 % ethanol for 1 h and complete drying was carried out by the Baltec Critical Point Dryer at Electron Microscopy Research Services at Newcastle University. After mounting, the coverslips were coated with 15 nm of gold using a SEM unit (Polaron Equipment Limited, Watford, UK).

2.10 Human Inflammation Antibody Array

All buffers and membranes were purchased from RayBiotch®, Norcross, USA. The buffers were prepared and stored according to the manufacturer's instructions. All the incubation steps were made at room temperature with rocking at 0.5 cycle/sec except the overnight incubations in which the samples stored at 4 ˚C in the fridge. The membranes were then incubated with 2 ml blocking buffer, supplied in the kit, for 30 min. The membranes were then incubated with 2 ml of conditioned media (spent media from Section 2.9) overnight at 4 °C. The media was discarded and the membranes were washed three times with wash buffer before further washes with buffer II. After washing, 1 ml of pre-prepared Biotinylated Antibody Cocktail was added to each membrane and incubated for a further 2 h. The washing steps were then repeated after discarding the Biotinylated Antibody Cocktail. A second wash was carried out before incubating the membranes with 1 X HRP-Streptavidin overnight at 4 ˚C. Final washes were made and the membranes were placed on tissue paper for drying and then transferred to a plastic sheet with printed side up. Detection buffer mix (500 µl/ membrane, 250 µl detection buffer C + 250 µl

detection buffer D) was added and another plastic sheet placed over the membranes with slight rolling to avoid air bubbles. The membranes were exposed to x-ray film within 10-20 min of detection buffer application and the best representive pictures were recorded at 1-2 min exposure time using highly sensitive Amersham Hyperfilm ECL, UK. The twenty different target cytokines are arranged in the array membrane as shown in Table 2.8.

Data analysis was made after subtraction the background values from reported spot. The final values were calculated using the following equation:

$X(Ny) = X(y) * P1/P(y)$

P1 = mean signal density of Positive Control spots on reference array

P(y) = mean signal density of Positive Control spots on Array "y"

X(y) = mean signal density for spot "X" on Array for sample "y"

X(Ny)= normalized signal intensity for spot "X" on Array "y"

Reference array= it is positive control spots to which the other arrays are normalized.

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	Eotaxin-1	Eotaxin-2	G-CSF	GM-CSF
2	POS	POS	NEG	NEG	Eotaxin-1	Eotaxin-2	G-CSF	GM-CSF
3	IFN gamma	IL-1 alpha	IL-1 beta	IL-2	IL-3	IL-4	IL-6	IL-7
4	IFN gamma	IL-1 alpha	IL-1 beta	IL-2	IL-3	IL-4	IL-6	IL-7
5	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13	I-309	TIMP-2
6	IL-8	IL-10	IL-11	IL-12 p40	IL-12 p70	IL-13	I-309	TIMP-2
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

POS = Positive Control Spot, NEG = Negative Control Spot, BLANK = Blank Spot

Table 2.8: The type and arrangement of inflammatory cytokines in RayBioteck array membrane.

2.11 Immunohistochemistry analysis

After alamarBlue® was applied, the tissue inserts from in-house constructed and MatTek models were transferred to new 6 well plate for washing with PBS, before 24 h fixation with 10 % (w/v) formalin. Tissue embedding and slide preparation was then carried out to prepare slides for further immunohistochemistry and immunofluorescence studies.

2.11.1 Haematoxylin and Eosin (H&E):

Staining with H&E was carried out for the in-house constructed and MatTek tissue models in the school of clinical dentistry/ the university of Sheffield and Royal Victoria Infirmary pathology laboratory/Newcastle Hospitals NHS Foundation Trust respectively using automated system (Ventana Benchmark Autostainer, Ventana Medical Systems Inc, USA). Slide examination was carried out by light microscope to investigate any morphological changes after amitriptyline exposure at all time points.

2.11.2 Apoptotic activity:

Two types of apoptosis antibody (anti-caspase 3 antibody) were used to investigate apoptosis throughout the 3D tissue model in response to amitriptyline exposure: Menapath polyclonal Caspase 3 antibody (Biocare LLC, California, USA) and a rabbit monoclonal caspase 3 antibody from R&D systems 269518 (R&D Systems, Inc., USA).

Using the aforementioned anti-caspase 3 antibodies, tissue slides were treated in the Royal Victoria Infirmary pathology laboratory/Newcastle Hospitals NHS Foundation Trust.

The immunohistochemistry was carried out in the department of Cellular Pathology, Newcastle upon Tyne Hospitals NHS Foundation Trust. The sections of formalin-fixed-paraffin-embedded 3D cell models (4 µm thickness) were immunohistochemically stained on a Ventana Benchmark Autostainer (Ventana Medical Systems Inc, USA). The primary antibodies for caspase 3 were used at 1:1000 and 1:300 dilution for rabbit monoclonal and menapath polyclonal respectively. The sections were subject to heat retrieval

with pH 9 buffer (Ventana Benchmark Autostainer protocol CC1) and staining was generated using ultraView detection reagent (Ventana Medical Systems Inc, USA). Sections of tonsil were used as positive controls, negative controls omitted the primary antibody.

Slides examination and counting was carried out using light microscope and the counting was made at 20 X magnification before it further calibrated by another researcher.

2.12 Immunofluorescences investigation of amitriptyline passage through 3D models

Both 3D model types were fixed with 10 % (v/v) formalin for 24 h after viability studies were carried out. Immunohistochemistry experiment was carried out investigating the presence of amitriptyline within the tissue using the primary antibody, mouse monoclonal anti-amitriptyline antibody (AbCam®, UK). Slides were washed with TBS-1 (20 µM), TBS-T (TBS-1+ 1% (v/v) Tween) and TBS-1 for 10 min for each step. Slides were incubated with primary antibody with 1:100 dilution in Triton™ (Sigma, UK) in humidifier containers for 24 h at 4 °C. After incubation, slides were washed before adding 250 µl of the secondary antibody (anti-mouse, Alexa Flour 488, from Molecular Probes®, Invitrogen, UK) in 1:500 dilutions in PBS. After 1 h incubation in the dark at room temperature, the washing was repeated; these steps were carried out in the dark. After washing, slides were mounted in medium of nuclei staining (Vectashield® HardSet™ mounting medium with DAPI, Vector laboratories, UK) plus glycerol before placing the cover slip. Examination was carried out using an Olympus BX61 microscope, Japan.

TBS-1: Tris Buffered-Saline 1X

TBS-T: Tris Buffered-Saline 1X-Tween

2.13 RNA extraction

RNA was extracted from monolayer cell lines (OKF6-TERT1) and human gingival tissue biopsy.

2.13.1 Monolayer cell culture

Trizol® reagent (Invitrogen, UK) was used to extract RNA, DNA and protein from monolayer cell culture. 1 ml of Trizol reagent was added to a T75 flask after discarding the culture media and the cell was washed with PBS. A cell scraper was used in the fume hood until the chalky homogenous mixture result was obtained. The homogenised sample was then incubated at room temperature for 5 min before addition of 0.27 ml of chloroform with vigorous shaking for 15 seconds. After 15 min incubation at room temperature, the sample then was centrifuged at 12,000 × g for 15 min at 4 °C in order to complete phase separation.

The aqueous phase supernatant, after centrifuging, was transferred to new tube, then precipitated by adding 0.625 ml of 100 % (v/v) isopropanol and incubated for 10 min at room temperature. The aqueous phase was then centrifuged at 12,000 × g for 10 min at 4 °C.

The supernatant was removed and the RNA pellet was washed by 1.25 ml of 75 % (v/v) ethanol and centrifuged at 7,500 × g for 5 min at 4 °C. The supernatant was removed and the tube left open at room temperature to dry for 5 min. The RNA was re-suspended with 20 µl of RNase-free water. RNA concentration was assessed using the Nano-drop spectrophotometer (Thermo scientific, UK) and the 280/260 ratio was recorded and RNA stored at -80 °C freezer, for downstream applications.

2.13.2 Gingival tissue

Trizol® reagent from Invitrogen, UK was used to extract RNA. Gingival tissue was kept in RNAlater® (Ambion®, Paisley, UK) at -80 °C after surgery and before use. After thawing, the tissue was weighed and collected in 1.5 ml micro-centrifuge tube before the addition of 0.75 ml of Trizol Reagent/50-100 mg tissue and pounded with pellet pestles for 5-10 min at room temperature to

homogenize the sample. Samples were then vigorously shaken for 15 seconds, after addition of 0.2 ml of chloroform. Before centrifuging, samples were incubated for 5-15 min at room temperature. Samples were then centrifuged at 12,000 × g for 15 min at 4 ˚C to complete separation.

The aqueous colourless upper phase, containing the RNA, was removed and employed in isolation, washing and re-suspension RNA extraction procedure. Intermediate and lower phenol phase was kept for future protein and DNA extraction procedures.

At new tube, aqueous phased was mixed with 0.5 ml of 100 % isopropanol. After 10 min incubation at room temperature, then it was centrifuged at 12,000- × g for 10 min at 4 ˚C to precipitated RNA as a gel like pellet.

Isopropanol supernatant and the pellet were washed with 1 ml of 75 % (v/v) ethanol. Samples were centrifuged at 7500 × $_g$ for 5 min at 4 ˚C. RNA pellet were air dried for 5-10 min and dissolved with 20 µl of sterile distilled water. Before RNA was stored at -80 ˚C, concentrations were checked by Nano-drop spectrophotometer (Thermo scientific, UK) by measuring the absorbance on Nano drop and 260/280 ratios were recorded.

2.14 RNA procedure

2.14.1 cDNA first strands synthesis

First strand of cDNA were prepared from RNA using reactions consisting of 1 µg of RNA, 1 µl Oligo (dt) (VH Bio Ltd, 50 µM) with RNase free water until a final of 12.5 µl. The samples were then incubated in thermostat reactions at 65 ˚C for 5 min, followed by 1 min incubation in ice before addition of 1 µl of reverse transcriptase enzyme (Promega, UK Ltd), 2 µl dNTP, and 5 µl of 5 x First Stand Buffer and 4 µl water. The whole volume then incubated at 50 ˚C for 60 min then at 70 ˚C for 15 min. For negative reaction, water was used in place of reverse transcriptase enzyme.

2.14.2 Polymerase chain reaction

A PCR reaction containing 1 µl template cDNA, 0.2 µM forward primer, 0.2 µM reverse primer, 12.5 µl Taq master mix (New England Biolabs, UK) and 10.5 µl water were used for cDNA amplification. PCR reaction cycle started at 94 ˚C for denaturation for 30 sec, extension at 72 ˚C for 60 sec over a course of 30 cycles. Annealing temperature was depending on primer design options but mainly ran between 50-62 ˚C for 30 s.

2.14.3 Agarose gel electrophoresis

Agarose gel was used to analyse PCR products. Agarose powder (Melford biolaboratories, UK) 1.5 % (w/v), dissolved in 50 ml of 1× TBE buffer (89 mM Tris base, 89 mM Boric Acid, 2 mM EDTA) and heated for 3-5 min avoiding excessive boiling. Gel red 10 % (v/v) was added to the cooled solution but before gelling and then poured into trays to set with gel combes in place. Eight microliters of PCR products were mixed with 2 µl of gel loading dye before placement in the gel slots. 5 µl of 100 bp DNA ladder was used to assess bands size were judged by 5 µl of 100 bp DNA ladder (Hyper ladder IV, Bioline, UK). Gels were run at 80 V for 1 h, and results were analysed and quantified by: G:BOX Chemi XL viewer; gene snap and gene tools software (SYNGENE, Frederik, USA).

2.14.4 Primer designs

Oligonucleotides primers were designed using Blast/NCBI data base web site or ordered directly from commercially available oligonucleotide options (Life Technologies Ltd, UK), Table 2.9. Primers were selected to contain 20-23 base pairs, 50-60 °C (annealing) temperatures and to span intron-to-intron junctions. Primers with G and C nucleotide at the 3 and 5 ends were preferred and GC content was not less than 50%. All primers were produced in Integrated DNA Technologies (IDT). Blast/NCBI data base web site: http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHom.

Name and accession no.	Primer sequence	Length (bp)	Annealing temp (°C)
ADORA2A NM_001278497.1	5'-$_{809}$TCCCCTTTGCCATCACCATC$_{828}$-'3 5'-$_{1414}$AGTCGGGGCAGAAGAAAGTG$_{1395}$-'3	606	58/60
GRIN1 NM_000832.6	5'-$_{1495}$AAGCCTCGAGGGTACCAGAT$_{1511}$-'3 5'-$_{1708}$TGCAAAAGCCGTAGCAACAC$_{1689}$-'3	217	50, 51.9, 53.8, 58 & 60
GRIN1* NM_000832.6	GGTCTGCAGGCTTCGCTCTAG GGCCCGGTCTTCCAGATGTT	490	52.3 & 54.8
GRIN2A NM_001134407.1	5'-$_{1123}$ GACAACAGCTTTGTGGGCTG$_{1141}$-'3 5'-$_{1337}$ TTGGGATGAGCTCCGTGTTC$_{1356}$-'3	235	50, 51.9, 53.8, 58 & 60
GRIN2B* NM_000834.3	5'-$_{271}$ CTCTTCTGAGAACGAGCTCTGCT$_{249}$-'3 5'-$_{777}$ CTCACTTTGTCTGGCCTTGCTTTC$_{753}$-'3	504	52.8 & 54.8
GRIN2C* NM_000835.4	CTCCAAGACCCAAGGCTGCTG ATGTGGCTCTCAGATGCCCTCTAG	510	54.9
GRIN2D NM_000836.2	5'-$_{643}$GTCCTTTGTAGCCGTGACCA$_{662}$-'3 5'-$_{1277}$TCCTGTCTCTGGTGAGGGAG$_{1259}$-'3	635	50, 53.8, 56.1 & 58
GRIN1 NM_000832.6	5'-$_{1495}$AAGCCTCGAGGGTACCAGAT$_{1511}$-'3 5'-$_{1708}$TGCAAAAGCCGTAGCAACAC$_{1689}$-'3	217	50, 51.9, 53.8, 58 & 60
SCN3A NM_006922	5'-$_{3174}$ CATTTTTGCTGTGGTCGGCA $_{3193}$-'3 5'-$_{3957}$ AGCTTGTTCACCTTCTCGGG$_{3938}$-'3	784	54, 56.4/53.1
SCN9A NM_002977.3	5'-$_{635}$ CCGTTTCAATGCCACACCTG$_{645}$-'3 5'-$_{1399}$GCTAAGAAGGCCCAGCTGAA$_{1379}$-'3	758	51.1
SCN11A NM_001287223.1	5'-$_{5824}$GCATCTTTGCCCTGGTAGGT$_{834}$-'3 5'-$_{1466}$GGCTGGTCTTTCCCAGACTC$_{1447}$-'3	643	53.1 & 51.1
CACNA2D1 NM_000722.2	5'-$_{671}$AGGCAGCCAGAGGATAAAACC $_{692}$-'3 5'-$_{991}$GATGTACCATGGTCTTCTGCG $_{970}$-'3	320	51.5/53.3

Table 2.9: Primers, sequences for all investigated receptors and ion channels. Subscript numbering refers to position of the primer on the gene. NCBI accession numbers included. * refers to commercial primers.

2.15 Purified PCR reaction from agarose gel

QIAquick Gel Extraction Kit (Qiagen, UK) was employed to prepare purified PCR reaction if multiple bands were found in agarose gel run. Bands of interest were excised using sterile scalpel blade with ultraviolet screen guide. According to the excised gel-piece weight, 3 volumes of Buffer QG were added to 1 gel volume as per manufacturer's instructions. It was then incubated at 50 ˚C for 10 min with intermittent mix by vortex until been dissolved. After gel completely dissolved, 1 gel volume of isopropanol (100 mg = 100 ul) was added, then the mixture was transferred to QIAquick spin column in 2 ml collection tube and centrifuged for 1 min at room temperature at 17,000 × g. After discarding the flow – throw a series of washing and centrifuge were made by adding 0.5 ml of Buffer QG, 0.75 ml of was added to column and centrifuged as previously. Further centrifuging was made before putting the spin column in new 1.5 ml micro-centrifuge tube. For DNA eluting, 50 µl of EB Buffer was added with further centrifuging to collecting DNA was sent for sequencing to confirm PCR product (Eurofins Genomics, UK).

2.16 Statistical analysis: Simple descriptive statistics, one-way ANOVA followed by Bonferroni *post hoc* tests, parametric independent sample t test and non-parametric two independent samples were conducted in SPSS version 22 (SPSS Inc., Chicago, IL).

Chapter: 3 Monolayer Cell Culture Experiments

3.1 Introduction

The journey to drug approval passes through several standard steps beginning with molecular planning which includes identifying the route of administration, target tissue and receptors. Following molecular planning the drug approval process continues through serial phases of safety and specificity where the drug under development will be tested regarding its function, metabolism and excretion (EMA, 2015b; FDA, 2015b). The drugs of interest in this study (amitriptyline, carbamazepine and gabapentin), are already approved as systemic medication, however, dose limits, changing the route of administration, and target tissue suggest different pharmacodynamics and pharmacokinetics. The drug regulatory authorities, like the FDA, is then not responsible for the safety of any compounded version of the drug for instance in a topical form for orofacial tissues (FDA). In any topical treatment, peripheral tissue and receptors are the sites of action for the drug with minimal to no systemic absorption. This means, therefore that cytotoxicity testing for the systemic administration of the drug may be misleading for the topical administration. In topical administration, cytotoxicity is mainly linked to tissue that is in direct contact with the applied medication and not to the systemic absorption process. It is therefore important to assess safety of topically applied medications at the level of the tissue they are in direct contact with, taking in consideration the use of high concentrations, that are usually implemented topically and the variation in tissue exposure times.

In this chapter, a group of investigations were carried out to assess the cytotoxicity of three neuromodulatory medications (NMs): amitriptyline, carbamazepine and gabapentin. These NMs were chosen as the initial drugs of interest as they have been identified as potentially helpful when applied topically in managing different neuropathic orofacial pain conditions (Padilla *et al.*, 2000). These NMs have been applied in various concentrations using varying application vehicles or carrier media such cream and Orabase paste (ConvaTec

UK, Flintshire, UK), (Padilla *et al.*, 2000; Sawynok, 2003; Heir *et al.*, 2008). None of these medications have, however, had their effects on cellular viability or attachment examined when applied topically to orofacial tissues. Cellular viability (including cell counting) and attachment is a crucial first step in decision making about drug safety (Niles *et al.*, 2008).

Safety and cytotoxicity testing in a monolayer cell line has been found to be convenient, cost effective, and reproducible when compared with animal or primary cell options, in both the drug industry and in dental material testing (Lovschall *et al.*, 2002). Using a monolayer cell line also helps to reduce, refine and modify animal involvement in drug discovery during preclinical testing (EMA, 1997). The types of cell line used in this chapter, were matched as closely as possible to the first cells in long-term contact with topical treatment in the orofacial region. Oral and skin keratinocyte, HaCat and OKF6-TERT cells respectively were used as relevant models to human skin and oral mucosa epithelial cells. These are immortalized non cancer cell lines. According to clinically prescribed topical treatments from the literature (Padilla *et al.*, 2000; Heir *et al.*, 2008), high concentrations of NMs were implemented in this chapter comparing with concentrations used previously in cytotoxicity studies investigating cytotoxicity of NMs.

Different types of cell based assays can be used to investigate cellular viability and cytotoxicity in testing drug safety. In the pharmaceutical industry, the biological and biochemical effect of drugs are submitted to automated cell based assay, in so-called “high throughput” screening. In this type of high throughput screening various assays are used to examine cellular viability including alamarBlue®, which is based upon biochemical cellular activity to reduce a chemical indicator (Resazurin) (Niles *et al.*, 2008). The investigation of cellular viability at different time points is helpful in deciding maximum exposure times and concentration safety. We utilised alamarBlue® in the experiments described in this chapter alongside a further reagent CCK-8 in a cell counting experiment. The reason we took two approaches to investigating the effect of NMs on cellular viability is that alamarBlue® may not actually reflect the number of viable cells as a few remaining viable cells may produce overlapping colour

change and the reverse is also true (Ramirez *et al.*, 2010). Cell-counting, therefore, serves as a useful cross-check of the results obtained from alamarBlue® testing.

To find concentrations of NMs to be released from the topical treatment carrying agent in clinical treatment, a clinical orabase paste was prepared in the laboratory with desired concentrations of NMs and high-performance liquid chromatography (HPLC) was used to find how much concentrations will release from topical NM and to be used in the morphology and cytokine expression studies later. The NMs employed may also impact upon cellular integrity through membrane integrity and cellular attachment, so this was examined through the use of a scanning electron microscope to scrutinise any changes in cellular attachment and shape. To explore the changes in the inflammatory status after NM exposure, a Human Inflammatory Antibody Array was implemented serving this goal.

3.2 Cellular viability after exposure to NMs

To optimize the viability methods to be used, several experiments with different concentrations were setup. The main objective was to apply the same high concentrations used clinically as topical treatment. However, problems were observed in dissolving the NMs in culture media using high concentrations, even when a low pH was induced. Carbamazepine solution preparations were associated with crystallization, which then interfered with the accuracy of plate reader readings.

Further methods were designed, trialled and modified including the application of high concentrations of NMs mixed with rat collagen gel, which was then used to coat the bottom of well plates, but this resulted in a turbid solution and interfered with viability measurement.

Clinical concentrations of NM containing paste were also prepared in the lab using Orabase paste from ConvaTec, Flintshire, UK, (containing: gelatin, pectin, sodium carbnoxymethyl cellulose, polythene and liquid paraffin). These preparations showed that it was impossible to apply the paste directly on the monolayer cell culture or grow cells on top of the paste as complete cell death was expected.

As an alternative to coat the bottom of wells with paste, the walls of wells were coated with the desired concentrations of NM in the paste prior to seeding the cells in the wells. The problems experienced after using this method were that: It was difficult to control the constant volume of media as it was absorbed by the paste; the long incubation at 37 °C caused disintegration of the paste which disturbed the culture media.

Following the experiences outlined above, a pragmatic decision was made to use a range of concentrations, diluted in media using the highest concentrations possible of the NM that still allowed full dilution and did not interfere with viability assessment. The cellular viability was measured using alamarBlue® as a colorimetric reaction, using two exposure methods which utilised two concentrations of NM (designated as high and low concentration) on three types

of cell line (3T3 mouse fibroblast cells, OKF6-TERT1 oral keratinocytes cells and HaCat skin keratinocytes cells).

The two exposure methods used were:

a) Cumulative NMS effect.

b) Specific time interval effect of NM.

3.2.1 Experimental results from exposure of cell lines to a cumulative NMs effect.

The three cell lines, 3T3, HaCat and OKF6-TERT1 were exposed to range of concentrations of the three NMs drugs. Ascending time points were implemented in this exposure method in an attempt to simulate the effect of continual cumulative exposure to NMs over time. The following results are for two sets of concentrations (high and low concentrations), the results of complete range of concentrations for the all NMs are presented in the Appendix B (Figure B.1, Figure B.2, Figure B.3, Figure B.4, Figure B.5, Figure B.6, Figure B.7, Figure B.8 and Figure B.9).

In the mouse fibroblast, 3T3 cells, exposure to amitriptyline in high concentration (1.8 mM) was associated with a significant reduction in cellular viability at all time points measured compared with untreated control cells ($p<0.05$, one-way ANOVA followed by Bonferroni's post hoc test). Low concentration of the same drug (200 µM) reduced cellular viability significantly after only 24 h exposure. At both concentrations of carbamazepine (100 µM low; 1.7 mM high) cellular viability was not affected at any time point ($p>0.05$). Both concentrations of gabapentin (150 µM low; 5.54 mM high) reduced cellular viability in the 3T3 cells: low concentration induced a significant reduction in cellular viability at all time points; high concentration was associated with a significant decrease in cellular viability reduction after 2 h exposure ($p<0.05$). The reduction in cellular viability with gabapentin was not as marked as seen in the amitriptyline exposure, especially at the higher concentration (1.8 mM) and it changed or reduced over time indicating possible cellular adaptation Figure 3.1).

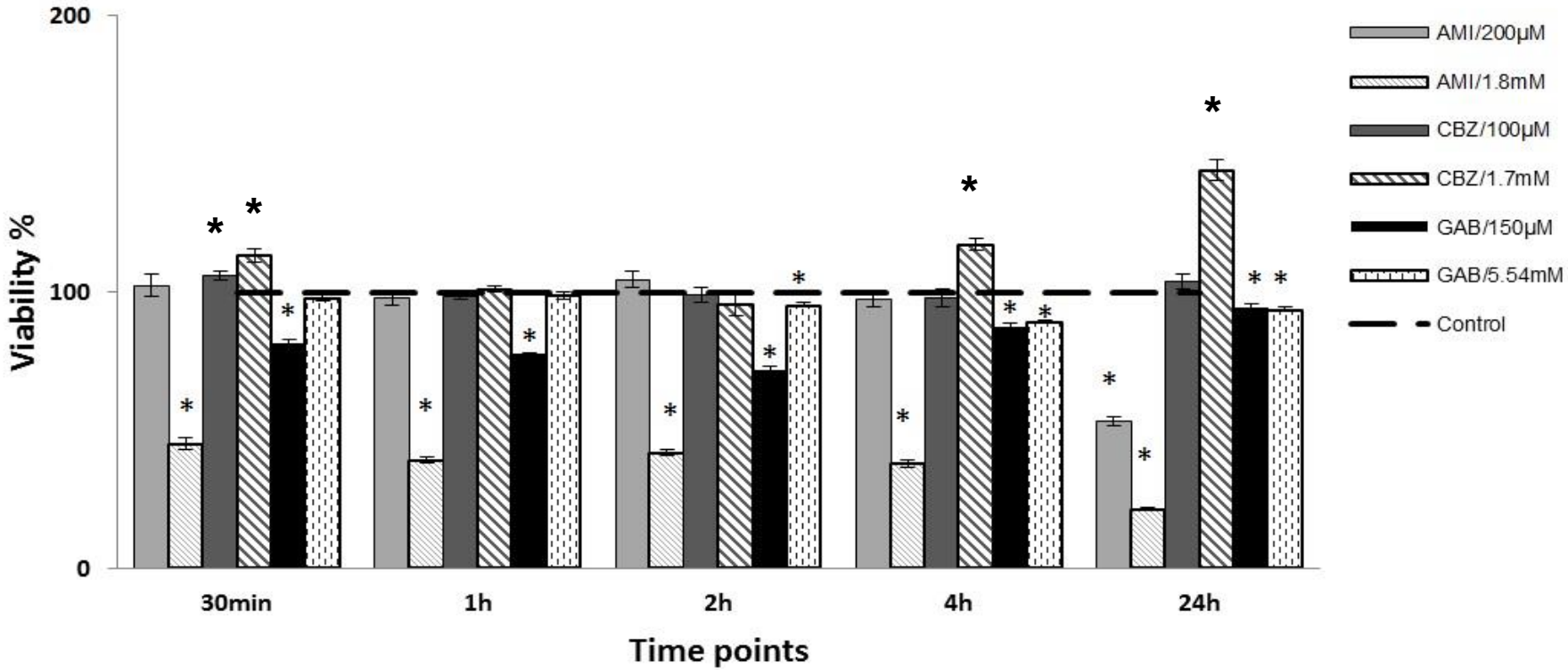


Figure 3.1: Cumulative cellular viability of 3T3 cells exposed to two different concentrations of amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

The cellular viability results for skin keratinocytes, HaCat cells, following exposure to the three NMs are shown in Figure 3.2. In these experiments, amitriptyline exposure produced a significant decrease in cellular viability in HaCat cells at high (1.8 mM) concentrations at all time points compared with untreated control cells ($p<0.05$). Low (200 µM) amitriptyline concentration induced a significant decrease in viability at 30 min, 1 h and 4 h exposure time points. The reduction in cellular viability observed with low concentration of amitriptyline was, however, smaller in magnitude compared with the difference observed between control and high amitriptyline concentration exposure.

A significant reduction in cellular viability in HaCat cells exposed to carbamazepine only occurred at high (1.7 mM) concentrations after 2 h of exposure ($p<0.05$). Gabapentin produced a significant reduction in viability at high (5.54 mM) concentrations at all time points compared to control ($p<0.05$). Non-significant changes in viability were found when HaCat cells were exposed to low concentrations of carbamazepine and gabapentin at nearly all time points, with the exception of 4 h exposure time point with low carbamazepine (100 µM) concentration ($p>0.05$).

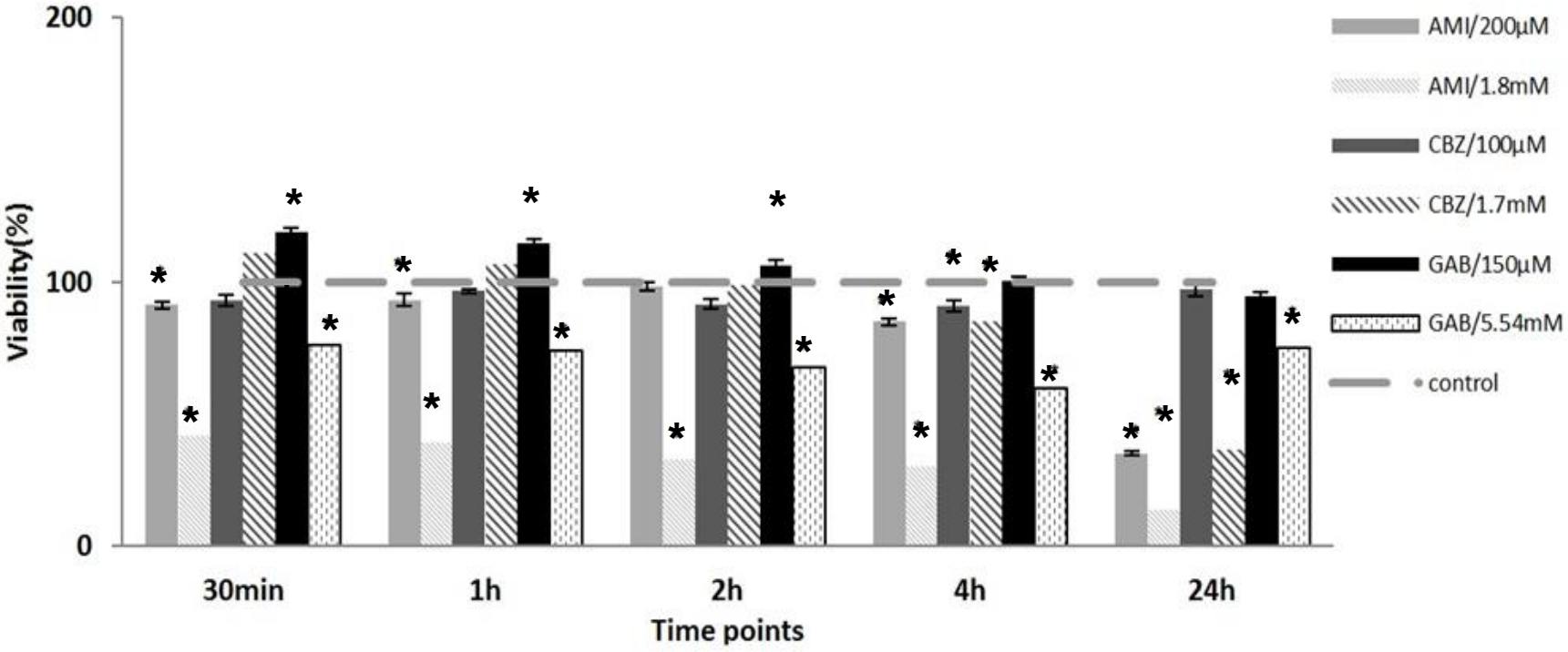


Figure 3.2: Cumulative cellular viability of HaCat cells exposed to two different concentrations of amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB), (indicated) measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

In oral keratinocyte cells, OKF6-TERT1, significant decreases in cellular viability ($p<0.05$) were only demonstrated after 4 h exposure to NMs, except for amitriptyline where high concentrations (1.8 mM) exposure revealed a significant decrease in cellular viability after 2 h exposure ($p<0.05$) comparing to control. A low concentration of amitriptyline (200 µM) caused significant reduction of viability after 4 h of exposure ($p<0.05$).

A significant decrease in cellular viability occurred after carbamazepine high concentrations (1.7 mM) exposure after 4 h compared to control ($p<0.05$), while low carbamazepine concentration (100 µM) exposure did not associate with significant decrease of cellular viability at all time points ($p>0.05$).

The only exception was gabapentin, where both high and low concentrations of gabapentin did not significantly affect cellular viability at all time points ($p>0.05$), the results are presented in Figure 3.3.

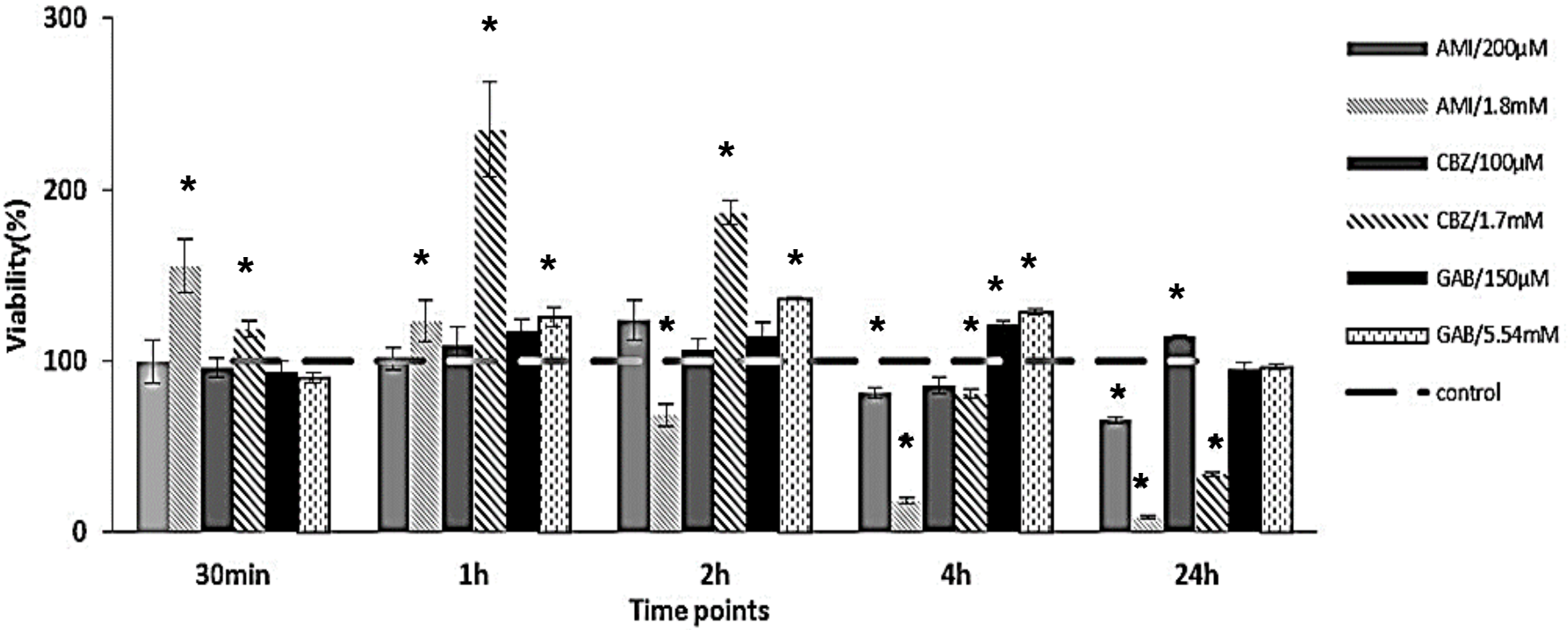


Figure 3.3: Cumulative cellular viability of OKF6-TERT1 cells exposed to two different concentrations of amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB), (indicated) measured using alamarBlue®. Mean values + SE (n=8) are shown for each experiment. Compared with untreated control cells by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

The viability results for the three NMs in all three cell lines treated demonstrated an extreme effect of amitriptyline on cellular viability which began after 30 min exposure time, and was especially marked with high concentrations (1.8 mM). Carbamazepine and gabapentin appeared to disrupt cellular viability less and even their reported significant reductions, may not be as meaningful clinically as the response of the cells to exposure of amitriptyline at both low and high concentrations.

Median lethal values were calculated at both 30 min and 24 h cumulative time points for each cell line and for each NM they were exposed to (Figure 3.4). Amitriptyline exposure was associated with a median lethal dose effect in all cell lines. The median lethal dose (LD_{50}) of amitriptyline in 3T3 cells was 1 mM at 30 min exposure and 158 µM at 24 h exposure time point. In HaCat cells, amitriptyline LD_{50} values were 512 µM and 186 µM at 30 min and 24 h time points respectively whilst in OKF6-TERT1, the only LD_{50} for amitriptyline was after 24 h exposure in which LD_{50} was 630 µM.

For the other NMs, the only LD_{50} value recorded was for carbamazepine in HaCat cells, and this was 398 µM after 24 h exposure time (Figure 3.5). Gabapentin did not demonstrate any LD_{50} effect in any cell line used at either of the two time points examined (Figure 3.6).

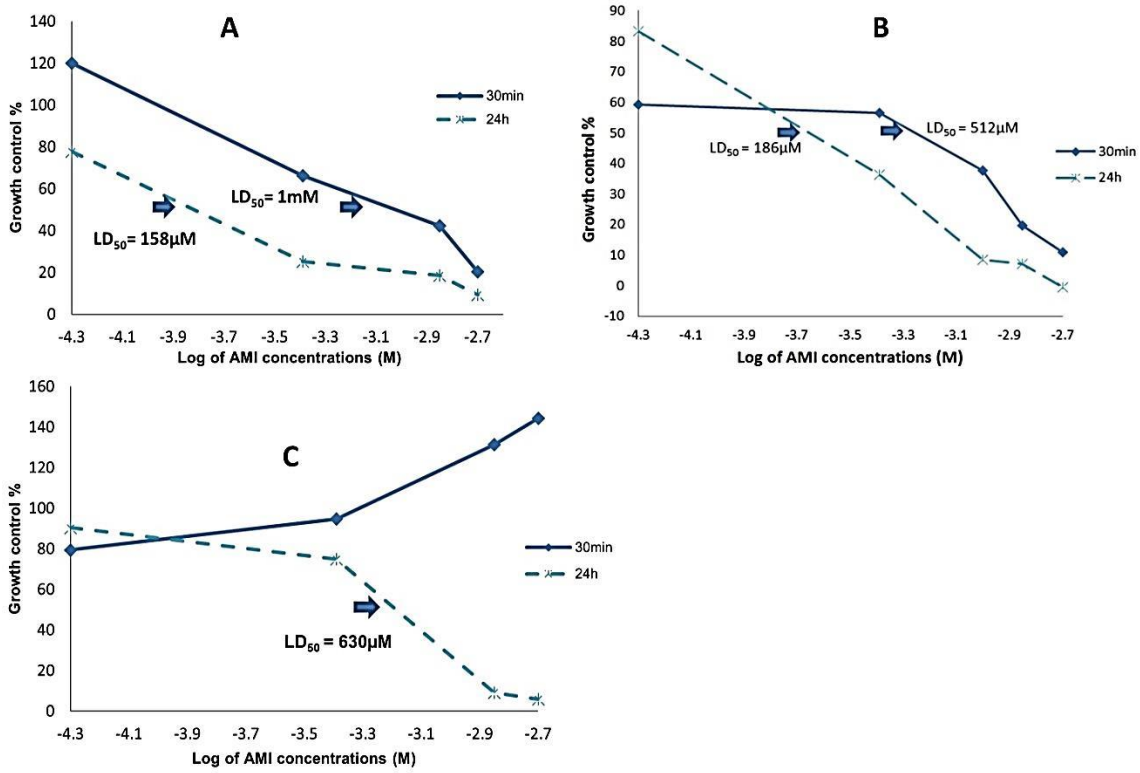


Figure 3.4: Median lethal dose (LD_{50}) calculation for amitriptyline (AMI) cumulative exposure at 30 min and 24 h in (A) 3T3 cells, (B) HaCat cells and (C) OKF6-TERT1 cells (n=8).

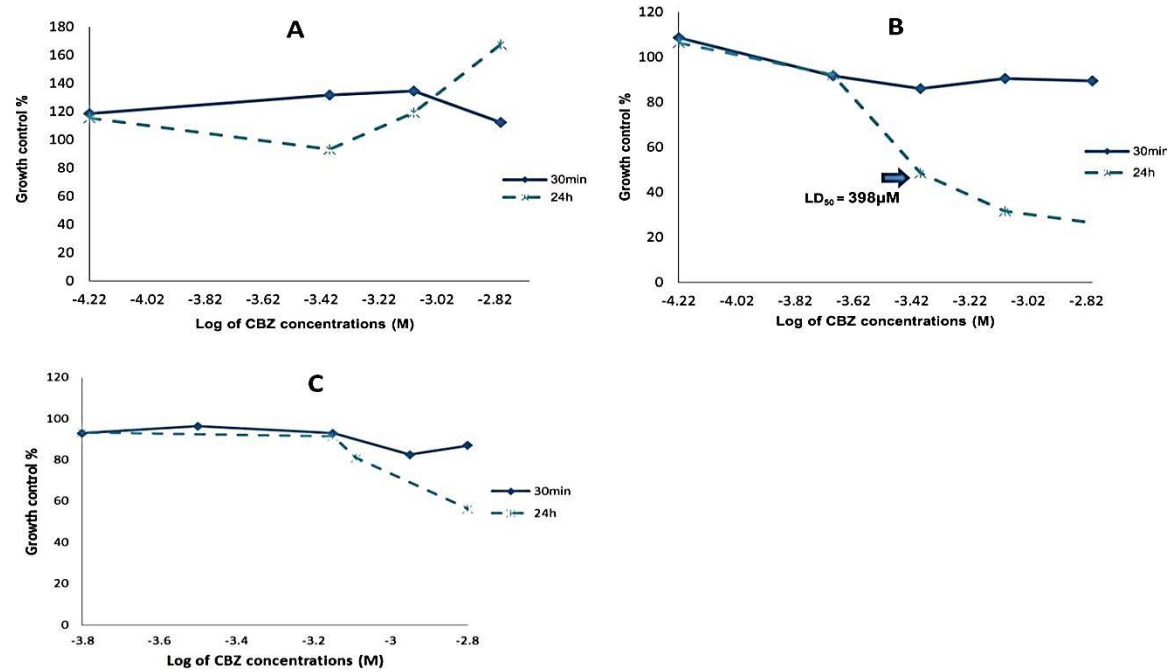


Figure 3.5: Median lethal dose (LD_{50}) calculation for carbamazepine (CBZ) cumulative exposure at 30 min and 24 h in (A) 3T3 cells, (B) HaCat cells and (C) OKF6-TERT1 cells (n=8).

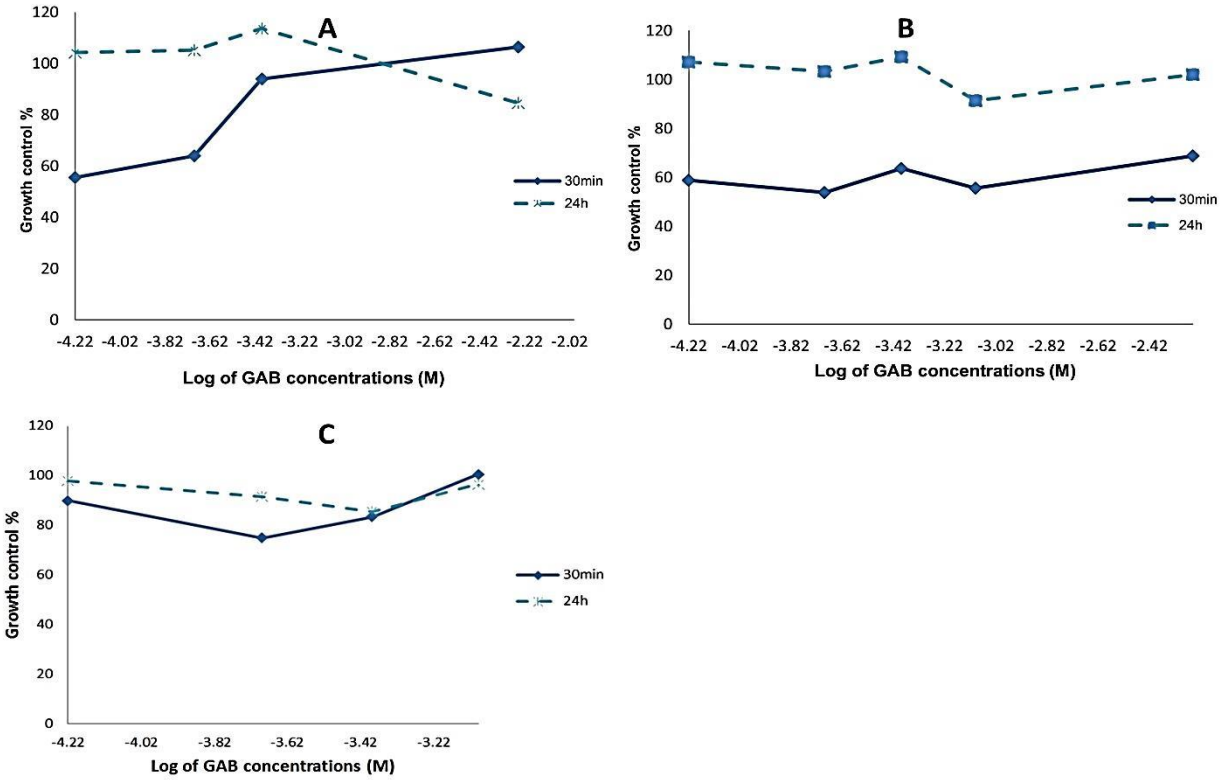


Figure 3.6: Median lethal dose (LD_{50}) calculation for gabapentin (GAB) cumulative exposure at 30 min and 24 h in (A) 3T3 cells, (B) HaCat cells and (C) OKF6-TERT1 cells (n=8).

3.2.2 Experimental results from exposure of cell lines to NMs for a specific time interval:

The same concentrations and viability assay were used in this method as in the previous section (3.2.1). Regarding the cell lines, only the oral keratinocyte (OKF6-TERT1) and skin keratinocytes (HaCat) cell lines were investigated, reflecting target peripheral orofacial tissue for topical treatment. In this method, only two time intervals for exposure to NMs were employed: 30 min exposure to NM, as this was felt to be as close as possible to the likely substantively of the NMs topical application in the oral cavity. A second, longer exposure time of 24 h, was used to represent the maximum possible exposure time of topical treatments.

A significant decrease in cellular viability in HaCat compared to untreated control cells was demonstrated at both time points (30 min and 24 h) and both concentrations (200 µM and 1.8 mM) of amitriptyline ($p<0.05$). In HaCat cells exposed to carbamazepine, a significant decrease in viability was only demonstrated at high concentrations of carbamazepine (1.7 mM) at the longer exposure time (24 h, $p<0.05$; Figure 3.7). Gabapentin showed less untoward biological effect in term of cellular viability with viability reduction reported as non-significant ($p>0.05$) compared to control at both time points and both concentrations.

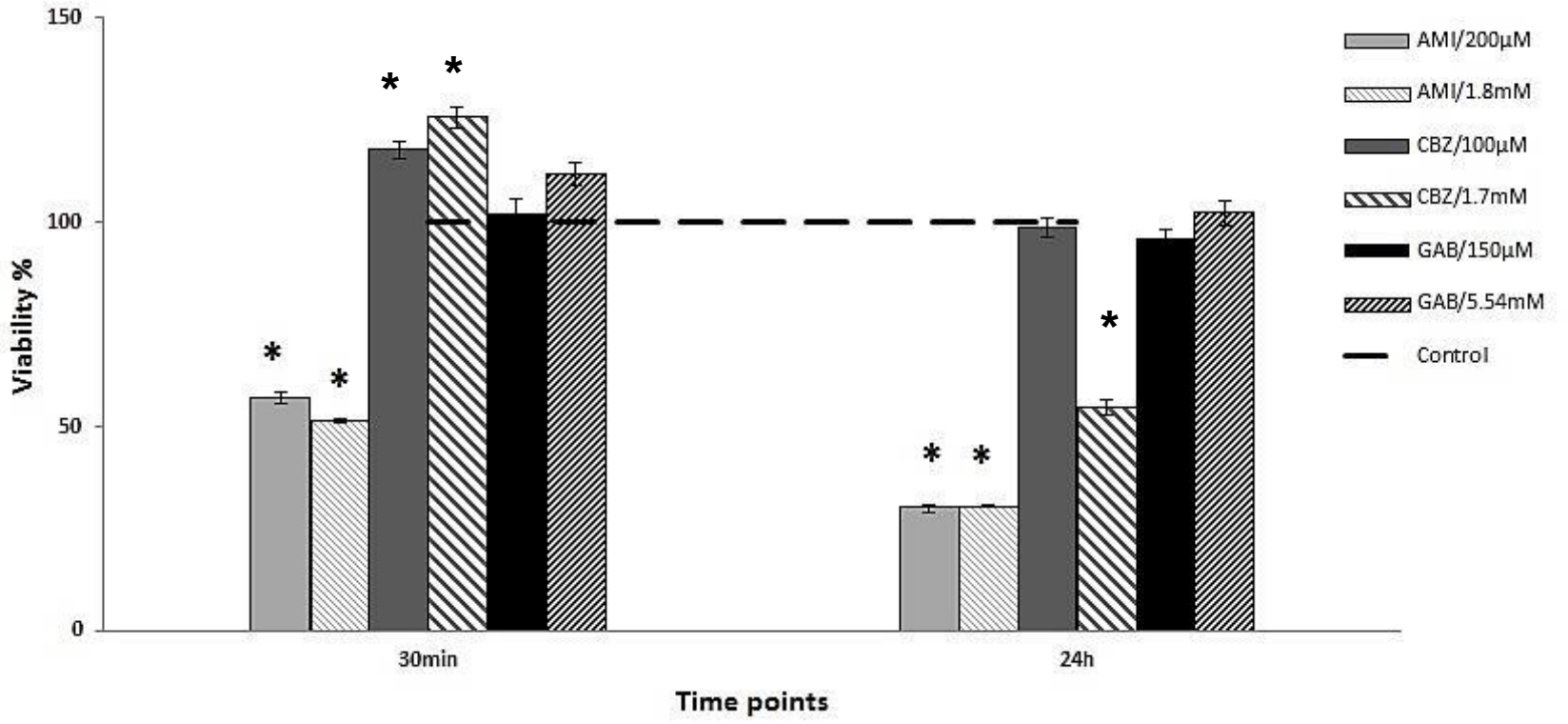


Figure 3.7: Specific time cellular viability of HaCat cells exposed to two different concentrations of amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at 30 min and 24 h time points, (indicated) measured using alamarBlue®. Mean values ± SE (n=6) are shown for each experiment. Compared with untreated control cells (dotted black line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

In OKF6-TERT1, as in HaCat cells, short and long exposure to amitriptyline at both low (200 µM) and high (1.8 mM) concentrations, resulted in a significant decreases in cellular viability compared with untreated control ($p<0.05$, Figure 8).

Carbamazepine and gabapentin induced a significant reduction in cellular viability ($p<0.05$) at low concentrations compared to untreated control at the short exposure time (30 min), whilst reduction in cellular viability was non-significant ($p>0.05$) at the long exposure time (24 h). Cells exposed to the high concentrations of carbamazepine and gabapentin demonstrated a significant reduction in viability at both time points (Figure 3.8).

The median lethal dose (LD_{50}) was calculated for 30 min exposure time only. The only LD_{50} value was 141 µM for amitriptyline in OKF6-TERT1 cells exposure (Figure 3.9). The other medications, carbamazepine and gabapentin, did not demonstrate any LD_{50} effect even at high concentrations (Figure 3.10, Figure 3.11).

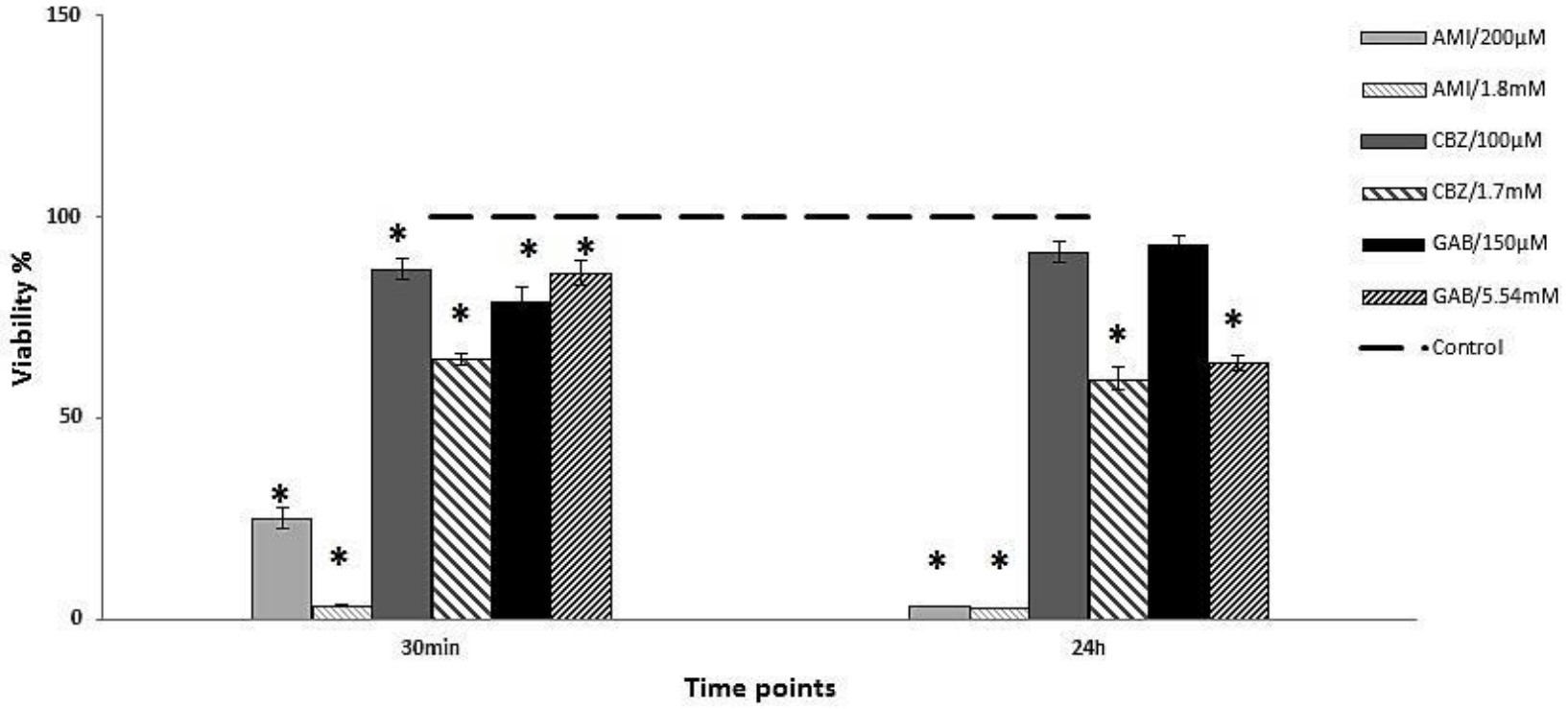


Figure 3.8: Specific time cellular viability of OKF6-TERT1 cells exposed to two different concentrations of amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at 30 min and 24 h time points, (indicated) measured using alamarBlue®. Mean values ± SE (n=6) are shown for each experiment. Compared with untreated control cells (dotted black line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

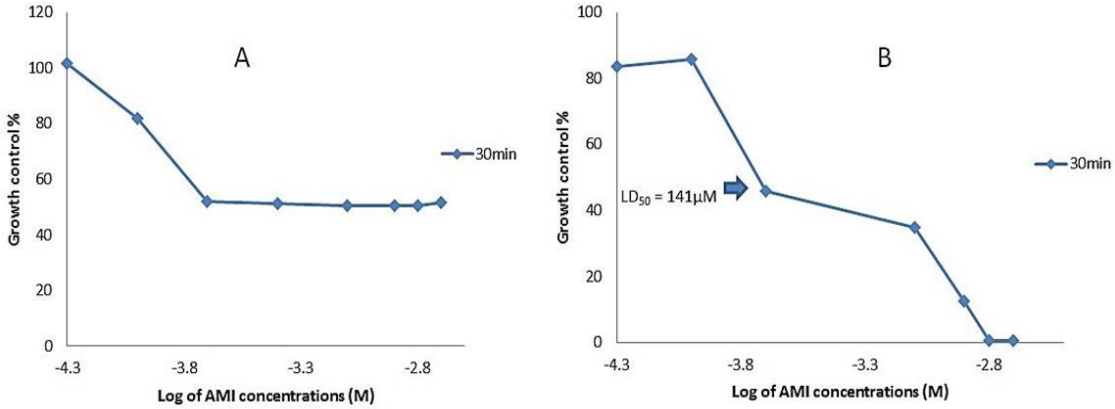


Figure 3.9: Median lethal dose (LD_{50}) calculation for amitriptyline (AMI) specific time exposure at 30 min in (A) HaCat cells and (B) OKF6-TERT1 cells (n=6).

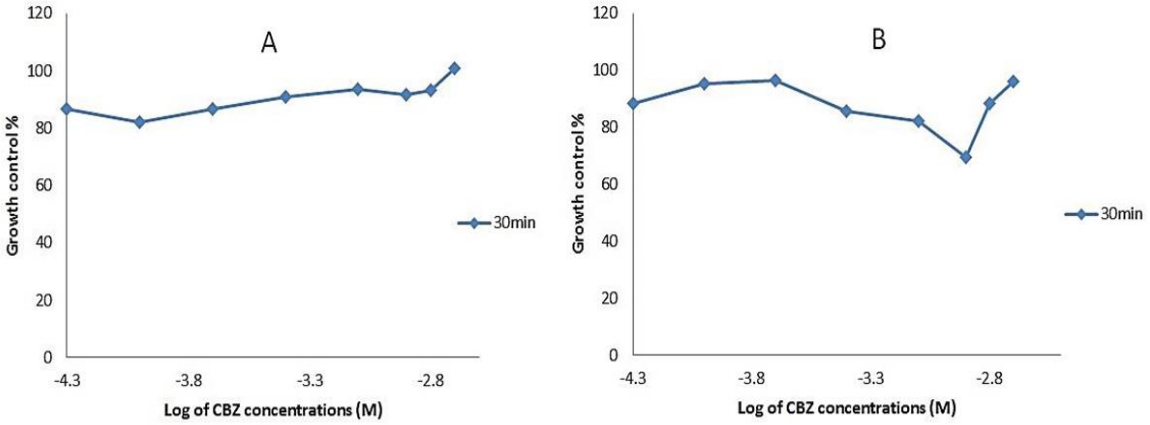


Figure 3.10: Median lethal dose (LD_{50}) calculation for carbamazepine (CBZ) specific time exposure at 30 min in (A) HaCat cells and (B) OKF6-TERT1 cells (n=6).

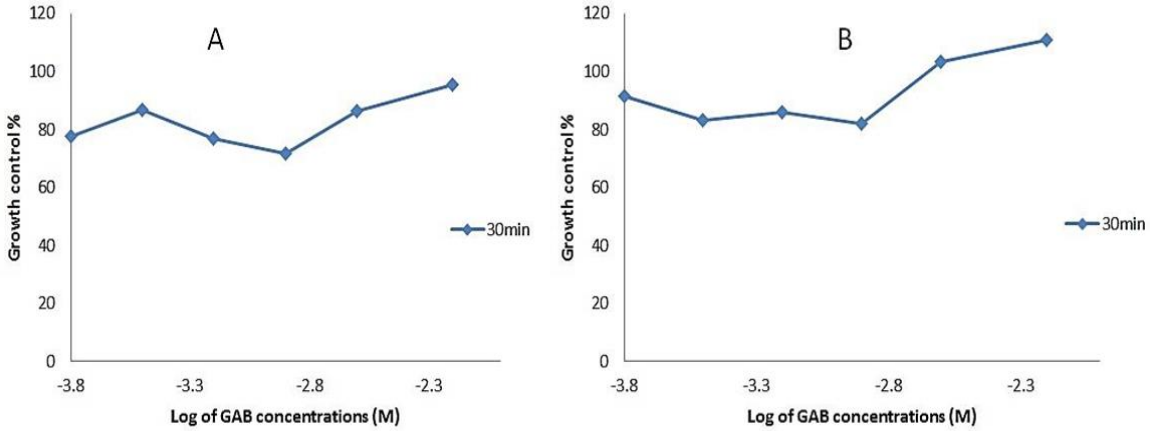


Figure 3.11: Median lethal dose (LD_{50}) calculation for gabapentin (GAB) specific time exposure at 30 min in (A) HaCat cells and (B) OKF6-TERT1 cells (n=6).

3.3 Cell counting assay:

Investigating cell counts in addition to examining cellular viability is essential in order to give a complete picture of the *in vitro* effect of NMs on target cells. This is due to the fact that chemical indicator changes measured in viability assessments using alamarBlue® occur due to a chemical reduction, which is affected by cellular activity and not necessarily number of cells. This can also change with exposure time and drug concentration. Cell counting in this group of experiments was carried out using Cell Counting Kit-8 assay (CCK-8). Only the keratinocyte cell lines, HaCat and OKF6-TERT1 were used for cell counting. A standard ascending serial count of untreated cells were seeded into a well plate and used to plot a calibration curve. The calibration curve was used as a reference point to estimate post NMs exposure cell count.

Compared to the calibration curve as a reference, the estimation of the number of HaCat cells was 2000 cells, after 30 min exposure to low (200 µM) and high (1.8 mM) concentrations of amitriptyline, about 20% of original seeding count (10,000 cells). A greater decline in the cell count occurred at the 24 h time point, where the number of cells counted was less than 1000 (10 % of original seeding count) for both concentrations of amitriptyline (200 µM, 1.8 mM); (Figure 3.12). Exposure to the low and high concentration of carbamazepine (100 µM, 1.7 mM) also demonstrated a decrease in the cell count after 30 min exposure (4000, 40 % of original seeding count). At 24 h of exposure to low concentration of carbamazepine, however, the cell count was similar to control cells. The high concentration of carbamazepine (1.7 mM) resulted in a drop in the cell count to less than 1000 cells (10 % of original seeding count); (Figure 3.13). Gabapentin exposure showed the least effect on the cell count with the main decline in cell count at 24 h exposure to the low concentration (150 µM) of gabapentin (9000, 90 % of original seeding count), (Figure 3.14).

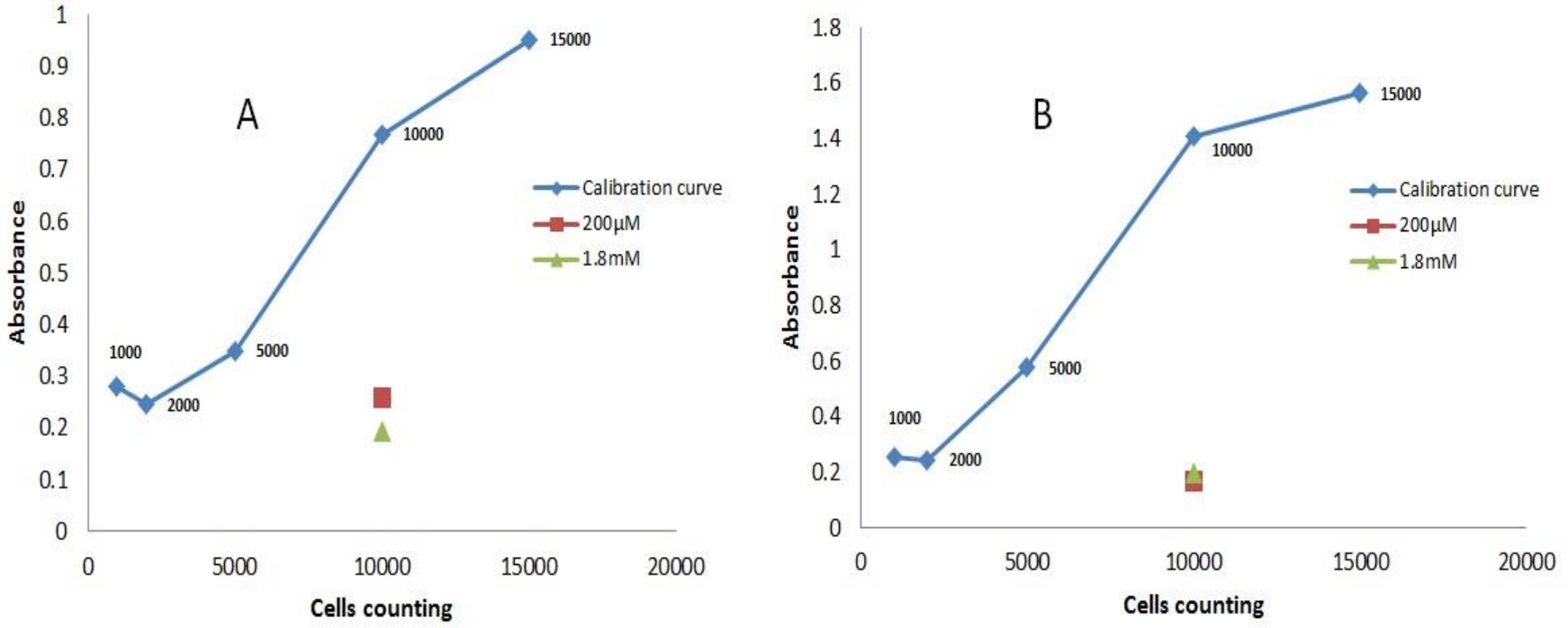


Figure 3.12: HaCat cell count after exposure to two different concentrations of amitriptyline at (A) 30 min and (B) 24 h time points. Cell counts were measured at an absorbance of 450 nm in the presence of tetrazolium salts (CCK- 8 assay). The calibration curve shows untreated cell counts at different densities of seeding. Mean values ± SE (n=6) are shown for each experiment.

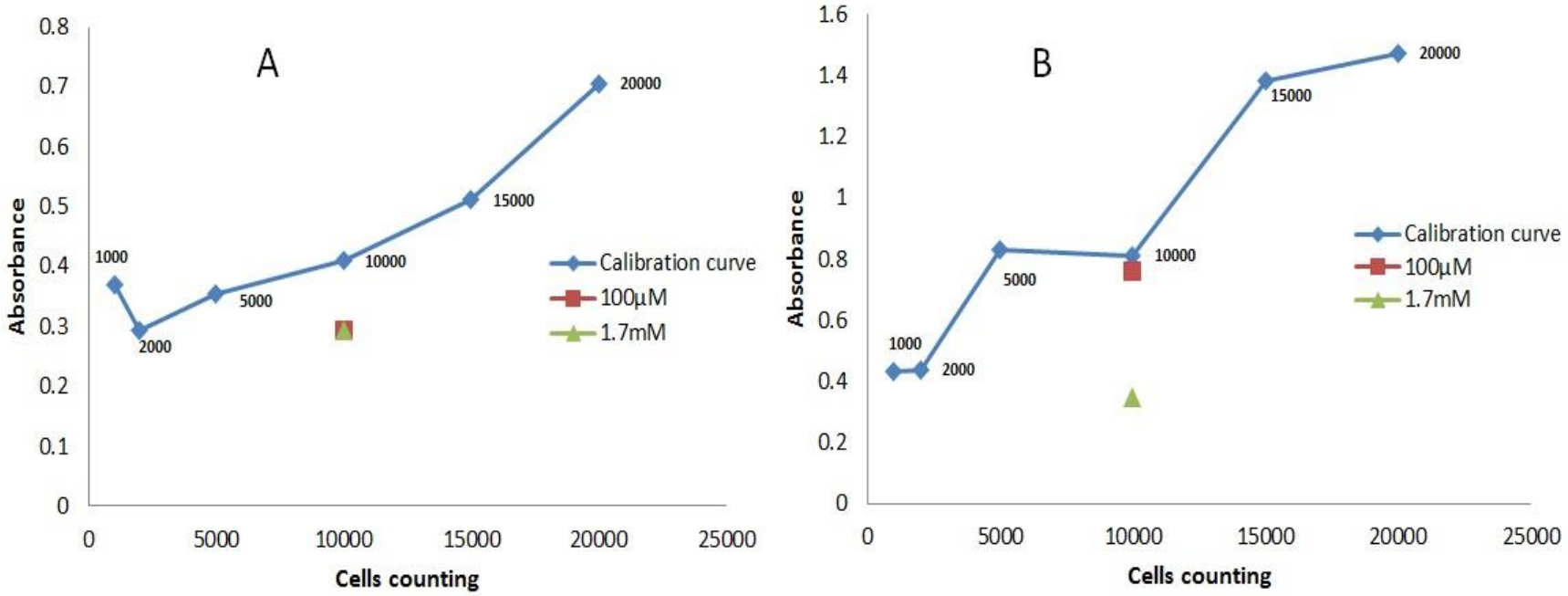


Figure 3.13: HaCat cell count after exposure to two different concentrations of carbamazepine at (A) 30 min and (B) 24 h time points. Cell counts were measured at an absorbance of 450 nm in the presence of tetrazolium salts (CCK- 8 assay). The calibration curve shows untreated cell counts at different densities of seeding. Mean values + SE (n=6) are shown for each experiment.

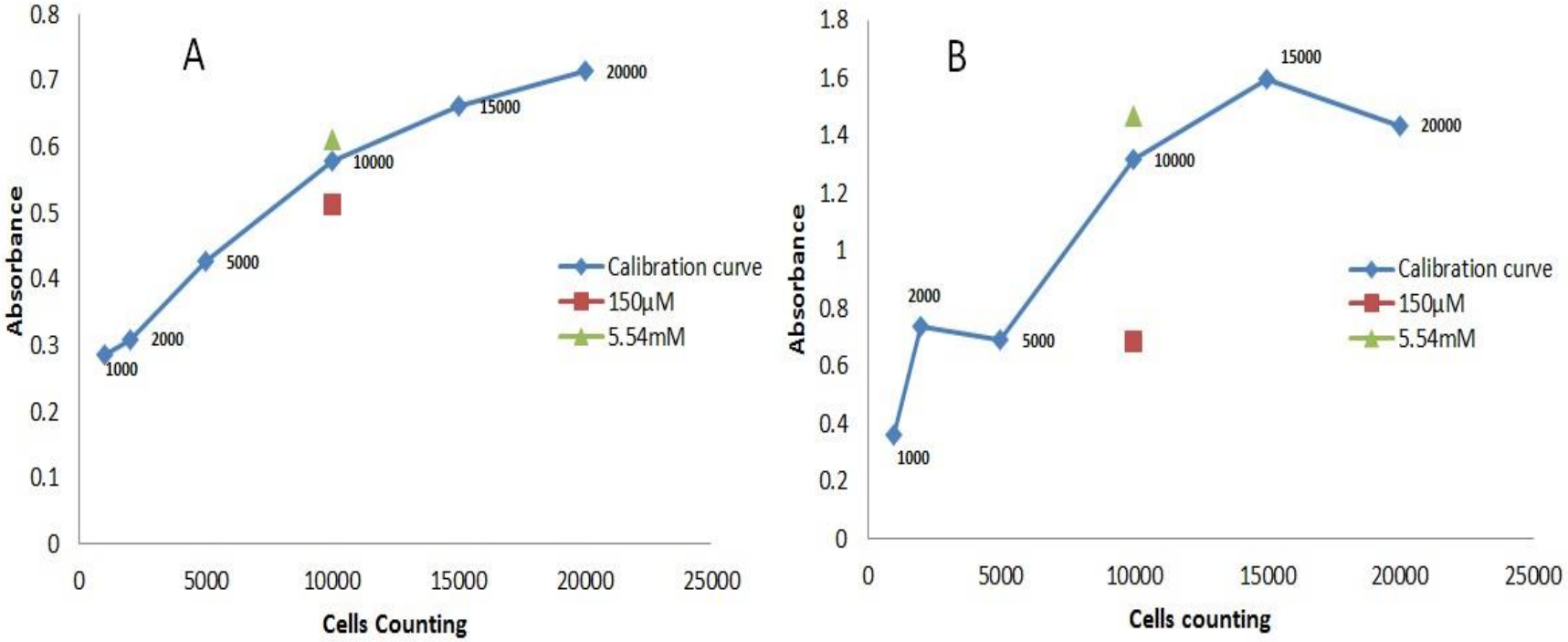


Figure 3.14: HaCat cell count after exposure to two different concentrations of gabapentin at (A) 30 min and (B) 24 h time points. Cell counts were measured at an absorbance of 450 nm in the presence of tetrazolium salts (CCK- 8 assay). The calibration curve shows untreated cell counts at different densities of seeding. Mean values ± SE (n=6) are shown for each experiment.

In OKF6-TERT1 cells, amitriptyline decreased the cell count at both low and high concentrations (200 µM, 1.8 mM) and both time points (Figure 3.15). After 30 min of exposure to low concentration amitriptyline (200 µM), the cell count decreased to 10,000, 50% of the original seeding count (20,000), whereas after thirty minutes exposure to the higher concentration (1.8 mM), the cell count decreased to less than 2000 cells, 10 % of the original seeding count. After 24 h of exposure, both concentrations of amitriptyline employed caused the cell count to decrease to less than 200 cells, 1 % of the original seeding count.

The short time exposure (30 min) to carbamazepine at high and low concentrations (150 µM, 1.7 mM) showed a slight effect on cell count, the estimated numbers were 18,000 (90 % of the original seeding count). After 24 h exposure, the cell count dropped to 10,000 cells (50 % of the original seeding count) for both high and low concentration solutions (150 µM, 1.7 mM), (Figure 3.16). Exposure to gabapentin did not cause a major reduction in the cell count (19,000, 95 % of the original seeding count) in comparison with the controls at either high or low concentrations (150 µM, 5.45 mM) at either time points (Figure 3.17).

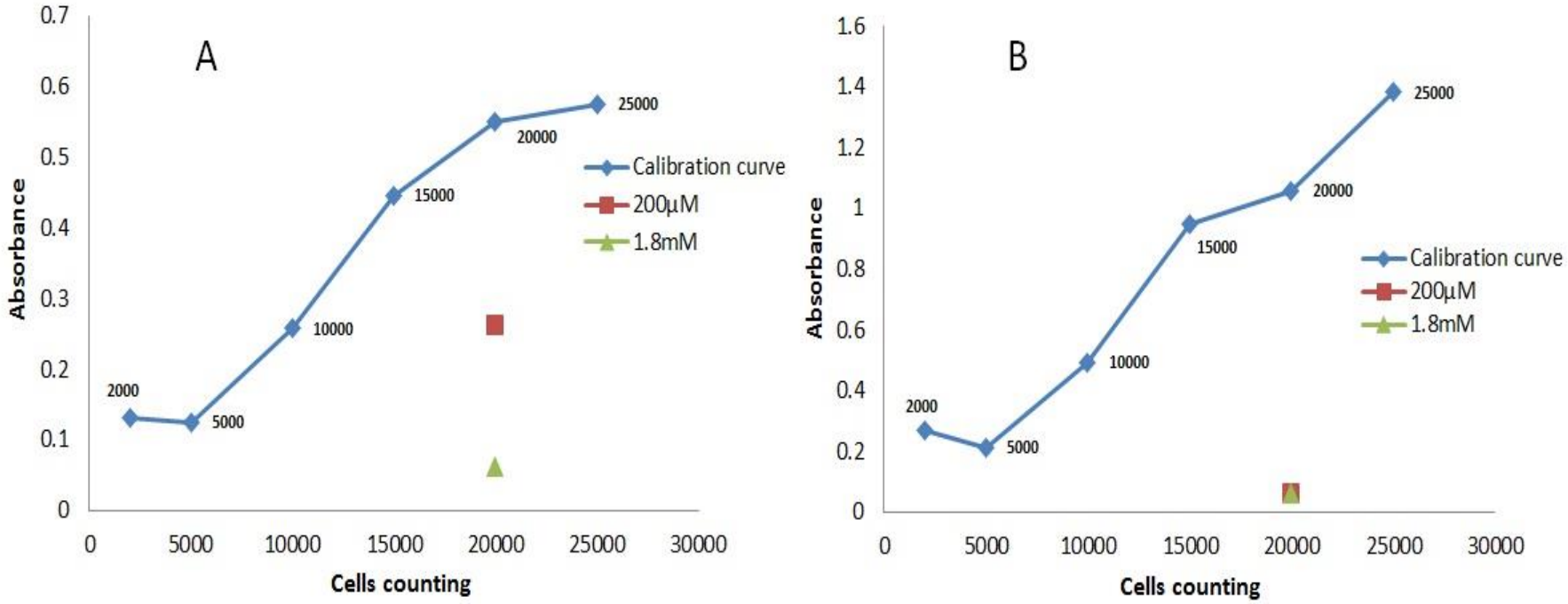


Figure 3.15: OKF6-TERT1 cell count after exposure to two different concentrations of amitriptyline at (A) 30 min and (B) 24 h time points. Cell counts were measured at an absorbance of 450 nm in the presence of tetrazolium salts (CCK- 8 assay). The calibration curve shows untreated cell counts at different densities of seeding. Mean values ± SE (n=6) are shown for each experiment.

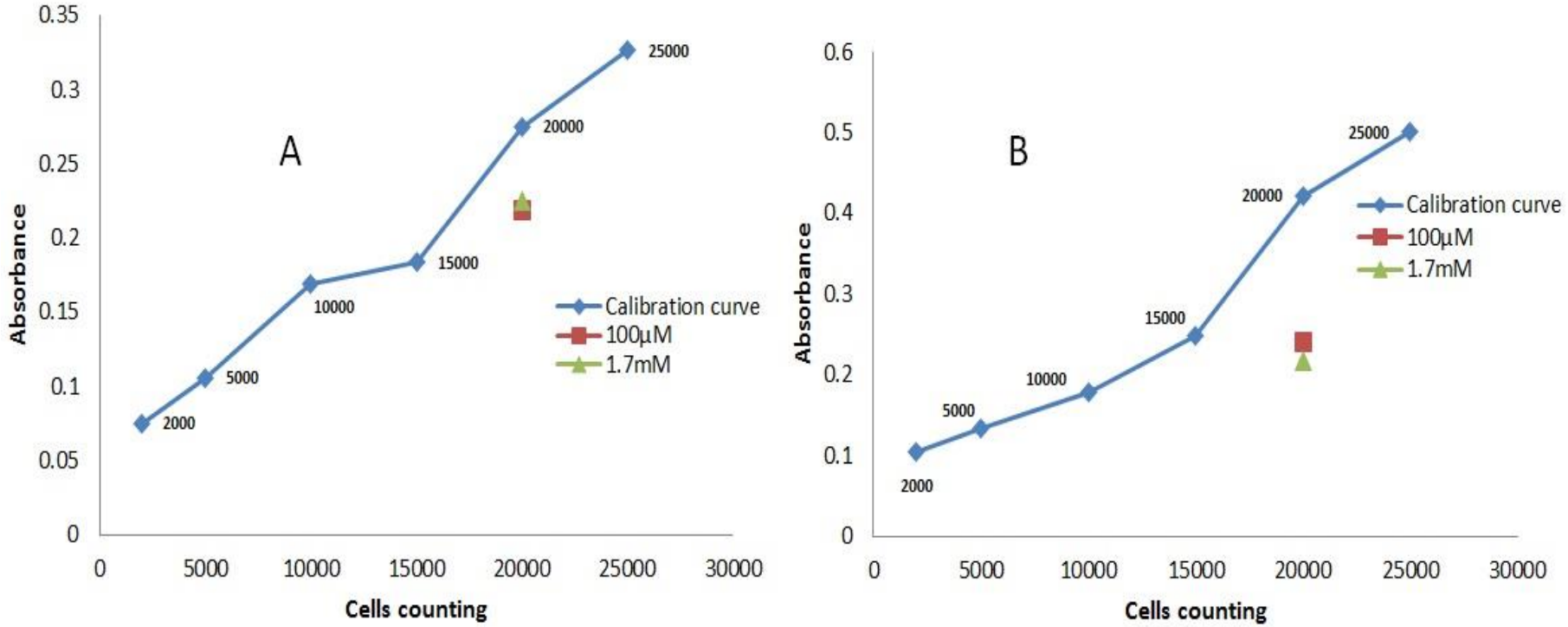


Figure 3.16: OKF6-TERT1 cell count after exposure to two different concentrations of carbamazepine at (A) 30 min and (B) 24 h time points. Cell counts were measured at an absorbance of 450 nm in the presence of tetrazolium salts (CCK- 8 assay). The calibration curve shows untreated cell counts at different densities of seeding. Mean values ± SE (n=6) are shown for each experiment.

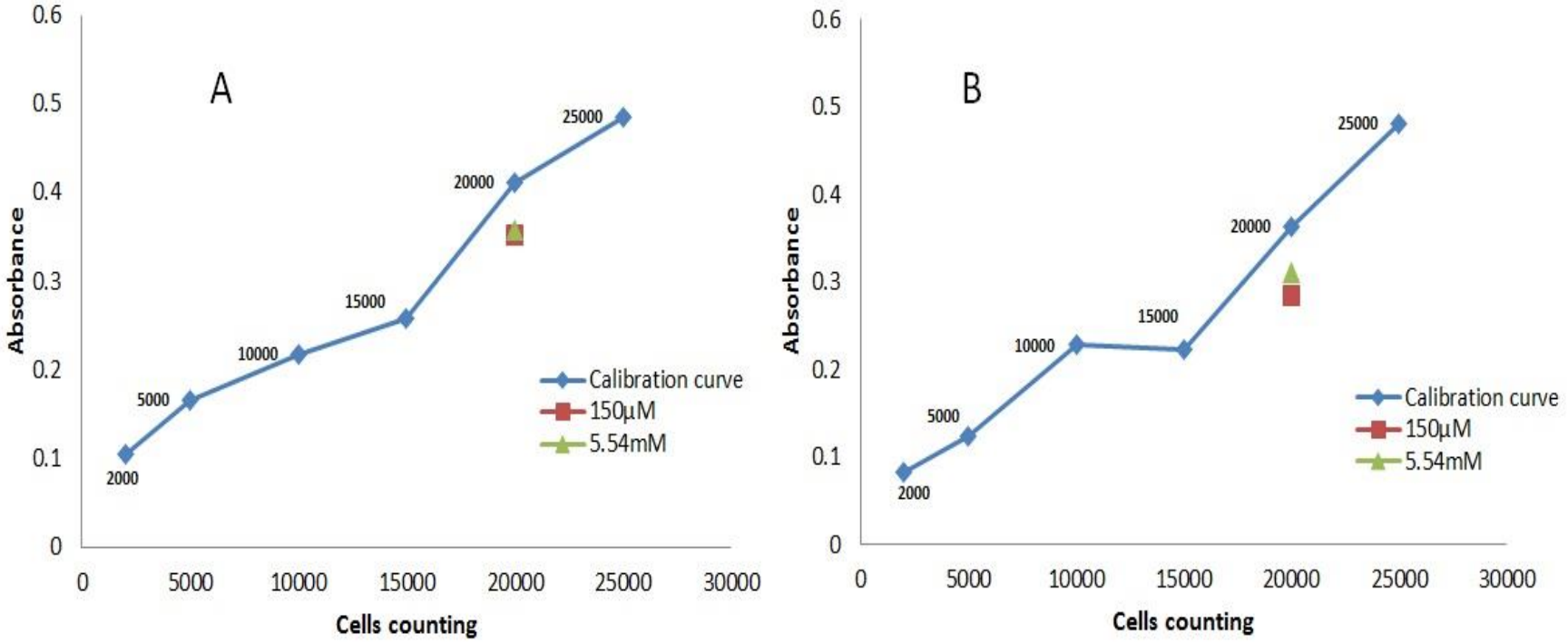


Figure 3.17: OKF6-TERT1 cell count after exposure to two different concentrations of gabapentin at (A) 30 min and (B) 24 h time points. Cell counts were measured at an absorbance of 450 nm in the presence of tetrazolium salts (CCK- 8 Assay). The calibration curve shows untreated cell counts at different densities of seeding. Mean values ± SE (n=6) are shown for each experiment.

3.4 Experiment to estimate the dosage of drug released from a standard topical paste.

Differing NM concentrations were tested in relation to their effects on cellular viability, but the concentrations employed in this chapter may not necessarily reflect the actual concentrations released from high concentrations of topical treatment which may be lower than the actual concentration in the topical carrying agent. As mentioned earlier in the chapter, I examined and explored a number of different options (Section 3.2), to directly test both higher concentrations of the pure drugs, and also carrier agent with clinical concentrations of the NMs, with cell-line models. Unfortunately, these differing options failed either through turbidity, crystallization, or immediate cell death thereby preventing accurate measurement of viability and cell count. The approach taken was therefore instead of merging both gel and cell culture at the same time, the paste was left in culture media for 30 min and 1 h respectively and the media were then examined to give an indication of the concentrations that may be released clinically (Hanasono *et al.*, 2004).

Clinical treatment in Orabase paste (PCP Direct online pharmacy, UK) was prepared in the laboratory, applying the most common clinical concentrations of NMs have been prescribed for orofacial neuropathic pain (Padilla *et al.*, 2000; Heir *et al.*, 2008; Haribabu *et al.*, 2013). The amounts released from these gels into the aspirated media were then investigated by HPLC analysis. Table 3.1, demonstrates the concentrations detected in the aspirated media of each NM after 30 min and 1 h. We then utilised the concentrations detected after 30 min in the aspirated media in the cellular morphology and cytokine expression experiments. These were thought to provide a closer representation of the likely clinical treatment application time.

Medications	Orabase paste concentrations % (w/w)	Released Concentration after 30 min	Released Concentration after 1 h
Amitriptyline	2 % (63 mM)	0.007 % (226 µM)	0.014 % (449.5 µM)
Carbamazepine	4 % (169 mM)	0.003 % (123.9 µM)	0.011 % (476 µM)
Gabapentin	4 % (233 mM)	0.09 % (5.54 mM)	0.18 % (10.5 mM)

Table 3.1: Orabase paste and released concentrations of amitriptyline, carbamazepine and gabapentin (w/w) are given along with molars concentrations. For released concentrations, results were determined by HPLC (n=2).

3.5 Cellular morphology and cytokine expression experiments

Using the short exposure time (30 min), two types of experiments were carried out to investigate the effect of NMs exposure on cellular morphology and cytokine expression:

3.5.1 Scanning Electron Microscope (SEM)

Prior to SEM 3T3, HaCat and OKF6-TERT1 cells were exposed to 30 min of the three respective NMs at the concentrations determined in Table 1 (AMI 226 µM; CARB 123.9 µM; GABA 5.54 mM). Cells were then prepared for SEM investigation as described in section 2.9. SEM images were made at two magnifications, 100 X and 1000 X, to investigate morphological and attachment changes. Careful examination of these images, revealed that exposure to amitriptyline for 30 min affected cellular attachment and morphology and resulted in rounding of both HaCat and OKF6-TERT1 cells indicating cell death and detachment (Figure 3.18, Figure 3.19). The amitriptyline exposure images were the only images from all three NMs to suggest extensive changes in the cellular attachment and morphology when compared with the control cell images. Mouse fibroblast (3T3) image results are presented in Appendix (Figure C.1), and confirm the same response to amitriptyline exposure. The exposure of HaCat cells to carbamazepine was associated with slight changes in morphology when compared to the control images and the OKF6-TERT1 cells response. Gabapentin exposure did not result in any obvious changes in morphology and attachment.

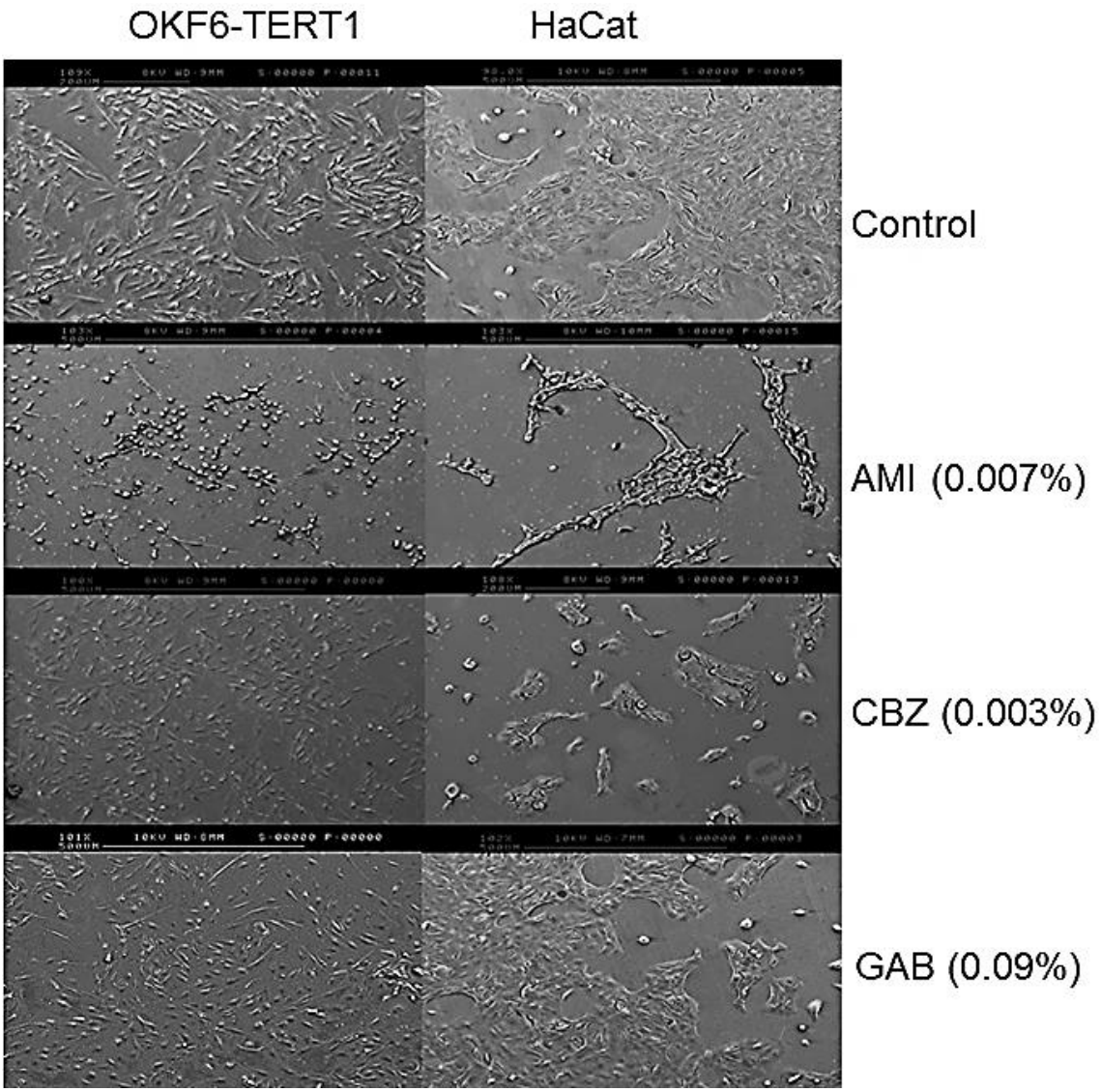


Figure 3.18: SEM images at 100 X magnification of OKF6-TERT1 and HaCat cells after 30 min exposure to amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at concentrations determined by HPLC. The top first panel of two images represents the normal morphology of control cells. The other images showing the changes after NMs exposure.

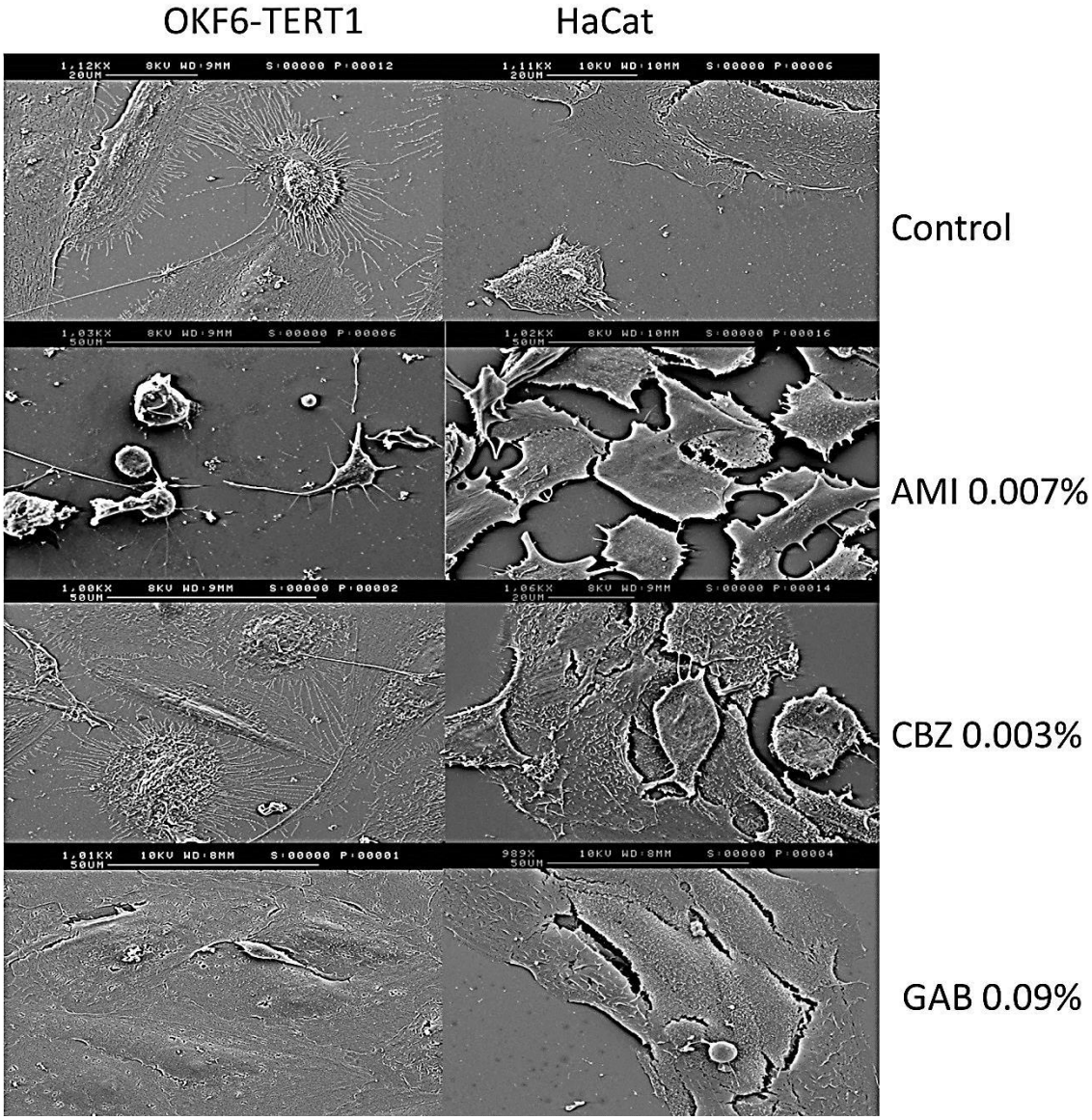


Figure 3.19: SEM images at 1000 X magnification of OKF6-TERT1 and HaCat cells after 30 min exposure to amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at concentrations determined by HPLC. The top first panel of two images represents the normal morphology of control cells. The other images showing the changes after NMs exposure.

3.5.2 Inflammatory cytokine release in response to NMs exposure

The expressions of twenty different inflammatory cytokines were investigated in HaCat and OKF6-TERT1 cell lines after exposure to the NMs at the concentrations determined by the drug release experiment that were also utilised in the SEM examination (Section 3.4 & 3.5). The exposure time of the cells to the NM was 30 min. The results of membrane experiments are shown in Figure 3.20 and Figure 3.21. The inconsistency of cytokine expression between the replicate experimental results indicated that there was no significant differences in cytokine expression between tested and control cells in both cell lines, however the main cytokine expressed was IL-8.

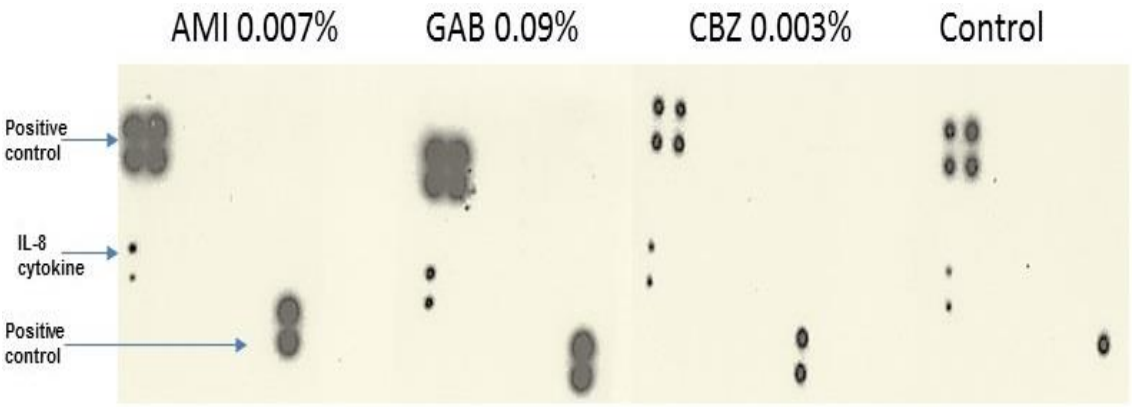


Figure 3.20: Cytokine expression after 30 min exposure of Skin keratinocytes, HaCat cells, to amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at concentrations determined by (HPLC).

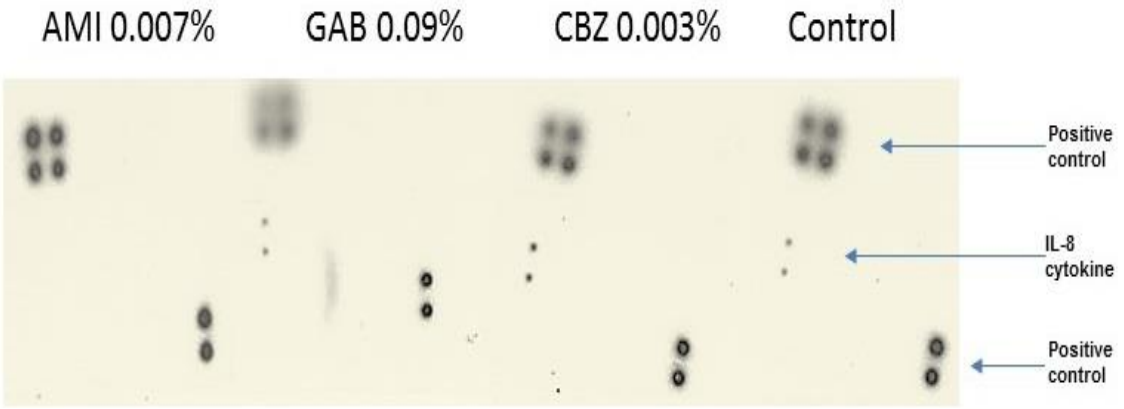


Figure 3.21: Cytokine expression after 30 min exposure of oral keratinocytes, OKF6-TERT1, cells to amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at concentrations determined by (HPLC).

3.6 Discussion and summary

Prior to commencing any Phase I clinical trial for a new drug, the U.S. Food and Drug Administration (FDA) requires detailed preclinical data regarding drug cytotoxicity investigation (FDA, 2015c). In these *in vitro* cytotoxicity studies, typical target tissues for systemically administered drugs have been examined including hepatic and neuronal tissues. In the case of topical compounded NMs used on orofacial tissues, given that the route of administration has changed from systemic use to topical, and the concentrations used have increased, these FDA previous approvals for safety and efficacy may no longer be valid in this context, so it is necessary to examine their toxic effects on orofacial epithelial tissues (Ahuja and Sharma, 2014; FDA, 2015a).

This *in vitro* study tested the effect of three commonly prescribed NMs, amitriptyline, carbamazepine and gabapentin, on cell lines culture. AlamarBlue® is a non-toxic colorimetric assay, it is reduced by mitochondrial processes making it a suitable assay for testing drugs known to cause mitochondrial dysfunction such as amitriptyline (Bopp and Lettieri, 2008). In addition, the alamarBlue® assay provides an accurate interpretation of cellular viability without killing the cells during analysis, and therefore allows for further studies and examinations to be conducted on the cells in question (Miret *et al.*, 2006; Al-Nasiry *et al.*, 2007).

The time points and exposure methods used, were designed to replicate, as far as possible the clinical course of topical treatment using NMs. Two exposure methods were employed: cumulative and specific time interval effects. The former method was designed to explore the different effects of exposure time of the target NMs. This is especially important as the topical treatment route is hypothetically combined with absorption into the tissues it is applied to. Depending upon the method of topical application, the time periods of exposure of these tissues to the NM may be increased. This is especially true in the clinical use of these types of topical NMs in the orofacial region as patients are often instructed to repeatedly apply them to the target tissue two to three times daily, perhaps even with a custom made appliance. The specific time-intervals examined incorporated a short exposure time (30 min), in order to demonstrate

the early effect of the applied medication on the surface cellular layer. The long specific time interval exposure time (24 h), was chosen to represent the longest possible exposure time or repeated application time that might be used by a patient. The change in viability as measured by alamarBlue® does not necessarily reflect the change in cell count, which is a known limitation of colorimetric assays. To ensure that cell count was taken into account, the change in cell count was assessed using the Cell Counting Kit-8 (CCK8; Dojindo Laboratories, Kumamoto, Japan) where the chemical indicator is different to that used in alamarBlue® that give comparable results and more confident in the reliability of the assays used than using the same test to find various parameters (Rampersad, 2012). The cell lines employed in these methods were: mouse fibroblast (3T3), in addition to skin keratinocytes (HaCat) and oral keratinocytes (OKF6-TERT1) as representative of orofacial tissue. Cell line application in toxicity investigations offers the first stages in cytotoxicity analysis, allowing multiple reproducible results with in minimal time, with reduced expense and time prior to experimentation with animal and primary cell studies (Barile *et al.*, 1994).

Amitriptyline cytotoxicity has been attributed to different mechanisms including: mitochondrial dysfunction; increased production of reactive oxygen species leading to alteration in cellular metabolism and permeability and elevated intracellular oxidative stress (Moreno-Fernández *et al.*, 2008; Cordero *et al.*, 2009). The *in vitro* cytotoxicity of amitriptyline has been investigated and reported previously in different human and animal cell lines, mainly constituting neuronal cell lines and hepatocytes as shown in Table 2. The most frequently used concentration of amitriptyline employed in these studies was the concentration measured at plasma level, where ingestion of a 100 mg tablet leads to approximately 0.15 µM - 0.7 µM plasma concentrations up to a maximum dose of 2.8 µM – 18.3 µM in the case of over dose and toxicity (Waller and Renwick, 1994b; Obuchowicz *et al.*, 2006). Higher concentrations were previously employed to study the apoptotic effect of amitriptyline on a cancer cell line, where Pula et al. (2013) found that 50 µM exposure after 24 h is enough to decrease cellular viability, with IC_{50} values of 4 µM after 48 h incubation. These studies illustrate the potential toxicity of amitriptyline in

different tissues and low LD_{50} value that jeopardize monolayer cell culture integrity.

In the current study, both concentrations of amitriptyline (200 µM, 1.8 mM) caused a significant reduction in the cellular viability in all cell lines even after a short exposure time. Exposure to amitriptyline decreased the cell count to less than 20 % and 10 % of the original seeding count in skin keratinocytes (HaCat) cell line at short and long time exposures, whilst in oral keratinocytes (OKF6-TERT), the cell count decreased to 50 % and 10 % of the original seeding count at short exposure time in low and high concentrations respectively before further deteriorating to <1 % of the original seeding count after 24 h. Scanning electron microscopy (SEM) confirmed these findings with obvious cellular changes in morphology indicating cellular death in all cell lines analysed. The cytotoxicity of amitriptyline was also demonstrated in its LD_{50} values, which were low compared with clinical concentrations used, in both cell lines and occurred at short exposure times.

The apoptotic and anti-proliferative effects of carbamazepine have been investigated and reported mainly in neuronal and glial cell culture, but also in blood cells, using plasma level concentrations up to 500 µM (Gao *et al.*, 1995; Awara *et al.*, 1998; Pavone and Cardile, 2003; Araújo *et al.*, 2004; Suwalsky *et al.*, 2006; Dambach *et al.*, 2014). Plasma levels of carbamazepine (13 µM- 50 µM) caused no effect on the viability of cancer cell lines (Ständer *et al.*, 1998). Compared with amitriptyline exposure, carbamazepine is reported to be non-cytotoxic except at higher concentrations, where the cytotoxic effect on cell culture was still less than that seen with amitriptyline exposure, using the same time points and cell lines (Mannerström *et al.*, 2006); (Table 2). Even IC_{50} value was not reported in most carbamazepine exposure in the same previous study, thereby corroborating our findings in these orally relevant cell lines, where carbamazepine exposure was found to be less cytotoxic compared with amitriptyline. Cytotoxicity was investigated on different cell types with higher concentrations (1.7 mM). In contrast to amitriptyline, carbamazepine was less cytotoxic in both cell lines, and the major changes in viability measured, only occurred at high concentrations and longer exposure times. The SEM findings

did not reveal any major changes in morphology and attachment compared with untreated control cells and compared with the gross changes found with amitriptyline exposure. The LD_{50} values were also confirmatory in this respect, as they were only calculable after a cumulative total of 24 h exposure which is not commonly applicable for the topical application of a gel in the intra or extra-oral treatment. The LD_{50} was 398 µM in HaCat cells after 24 h exposure.

Gabapentin appeared to be the least cytotoxic drug among the three NMs examined in this study. The previous studies found similar findings; in spite of the low concentrations they employed (Table 3.2). Even when significant viability changes occurred, they were mostly associated with high concentration and a long exposure time (24 h) and the amount of reduction in cellular viability and cell count was small compared with amitriptyline. There was also no LD_{50} calculable with SEM findings are not showing any recognizable cellular death signs.

Exposure of amitriptyline and carbamazepine at different time points and concentrations was associated with high plate reader readings and colour change of alamarBlue® compared with control (Figure 3.3 & Figure 3.7), which could either indicate an increase in cell number or an increase in ATP production by the viable cells. By using two different methodologies, and cell count using CCK-8 we were able to show that there was a decrease in cell counts at these time points and therefore we hypothesise that the result seen with alamarBlue® are indicative of increased cellular activity, which has been reported by Mannerström *et al.* (2006), in different cell lines after exposure to amitriptyline and carbamazepine.

OKF6-TERT1 exposure to carbamazepine and gabapentin showed change from a significant reduction at the 30 min time point to a non-significant change after 24 h time point. This could be explained as cellular adaptation to these drugs especially as the cell count did not change in the case of gabapentin at both time points and there was reduction in cell count after 24 h compared to the 30 min cell count.

The effect of NMs exposure on cytokine expression has been investigated previously in clinical studies and *in vitro* experiments. The exposure or treatment has previously been associated with a suppression of specific pro-inflammatory cytokines (IL-1β and TNF-α), in addition to over expression of anti-inflammatory cytokine (IL-10). Carbamazepine exposure and treatment has been associated with the over expression of the following groups of cytokines: (IL-2, IL-5, IL-6 and IL-10), (Obuchowicz *et al.*, 2006; Hashioka *et al.*, 2007; Backonja *et al.*, 2008; Mathieu *et al.*, 2011; Lee *et al.*, 2013). In our experiments, there was a very low level of detected cytokine expression with all NMs, and the cytokine expressions did not differ to untreated cells. This could be due to the short exposure time (30 min) used and the fact that a very crude method of measuring cytokine expression was employed. It allowed multiple cytokines to be analysed at the same time, however more sensitive measures, such as RT-qPCR or ELISA may have revealed different results. Amitriptyline cytotoxicity was reported in such short exposure as in the viability studies in this chapter, however, amitriptyline was found to induce apoptosis and cell death through its effect on mitochondria, subsequent release of caspase activating protein that causes activation of caspase 3 pathway and apoptosis (Moreno-Fernández *et al.*, 2008).

In summary, viability results, in both cumulative and specific time points, and cell count investigations gave consistently similar results for each NM tested. Amitriptyline exposure was associated with a reduction in cellular viability and cell count; carbamazepine and gabapentin exposure disturbed cellular viability and cell counts less, even at high concentrations. The SEM findings were consistent with the viability results with only slight changes and rounding cells in case of carbamazepine exposure.

Amitriptyline is well-documented as a topical treatment, and has been employed in many clinical trials, however, amitriptyline associated cytotoxicity has not been reported in any clinical trials relating to orofacial neuropathic pain or even in neuropathic pain affecting other parts of the body.

Before a decision to exclude amitriptyline from topical management of neuropathic orofacial pains can be made on the basis of safety, further

investigations using more sophisticated cell culture techniques is required. In the next chapter, we employ the use of a 3D tissue cell culture model, to carry out further experiments to strengthen the *in vitro* findings reported in this chapter.

Medications	Studies	Cell or tissue type	Exposure time	Concentrations		Viability %	Cell count %	LD_{50} or IC_{50} µM
				%	µM			
Amitriptyline	(Mannerström *et al.*, 2006)	HepG2[1] SH-SY5Y[2] U-373 MG[3] Primary mouse hepatocyte	24 h	0-0.003	0–100	<2% <3% <65% <20 (100 µM)		29.6 ± 4.3 48.1 ± 4.2 28.4 ± 3.1 7.2 ± 26.4
	(Dahlin *et al.*, 1999)	Primary endothelial Smooth muscle cells (Human)	0-2 h	0.0001-0.01	3-330	<90% 100 µM <20% 330 µM [4]		
	(Pula *et al.*, 2013)	HTB114[5]	1-7 days	0.00001-0.03	0.5-1000	<2% after 48 h at 100 µM		4 µM after 48 h
	(Lirk *et al.*, 2006b)	Primary dorsal root ganglion cells (Rat)	24 h	0.001-0.0047	50-150		<60 at 100 µM	
	(Moreno-Fernández *et al.*, 2008)	Primary skin fibroblast (Human)	48 h	0.0006-0.003	20-100		<6.5 at 100 µM	
	(Barile and Cardona, 1998)	HFL1[6] Detroit 551[7] WS1[8]	24 h					90 51 67
	(Boelsterli *et al.*, 1987)	Erythrocyte (Rat)	1 h					140 (HC_{50})[9]
	(Yang *et al.*, 2002)	HFL1	24 h					89 ±1.27

Medications	Studies	Cell or tissue type	Exposure time	Concentrations		Viability %	Cell count %	LD_{50} or IC_{50} µM
Carbamazepine	(Mannerström *et al.*, 2006)	HepG2 SH-SY5Y U-373 MG Primary Mouse hepatocyte	24 h	0-0.1	0–420	N <82 420 µM N <40		36.8±0.04 in mouse hepatocyte
	(Boelsterli *et al.*, 1987)	Erythrocyte (Rat)	1 h					140 (HC50)
	(Gao *et al.*, 1995)	Primary Cerebellar granule cells (Rat)	3 days	0.002	100	<40%		
	(Pérez Martín *et al.*, 2008)	Vero cells	4 h-24 h	0.0002-0.011	10-500	Sig at 100 µM after 24 h (Proliferation)		406.2 (Half maximal inhibition)
	(Valentine *et al.*, 1996)	cHol[10] h3A4v2[11]	67 H	0.005-0.02	211-846		<12.5 200 µM <28	
	(Pavone and Cardile, 2003)	Astrocyte primary cultures (Rat)	48 h	0.0001-0.01	0.42-42.3	<50 at 42.3 µM		
	(Regan *et al.*, 1990)	Neuroblastoma (neuro-2A) glioma (C6)	48 h	0.011-0.014	50-60	N		
	(Ständer *et al.*, 1998)	LN-229[12], LN-308[13], T98G[14] U87MG[15], U251MG[16], D247MG[17]	72 h	0.0003-0.00035	13-15	N		
Gabapentin	(Pavone and Cardile, 2003)	Astrocyte primary cultures (Rat)	48 h	0.0001-0.01	5.8-584	<72 at 584 µM		
	(Dambach *et al.*, 2014)	Primary glial cell culture (Rat)	24 h	0.001-0.01	85.4-584	N		

[1] hepatocellular carcinoma cell line, [2] neuroblastoma cell line, [3] malignant gliomas cell line, [4] after 2 h, [5] mesodermal tumour (mixed) cell line, [6] human lung fibroblast cell line, [7] Skin fibroblast cell line, [8] skin fibroblast, [9] drug concentration causing 50% haemolysis, [10] B lymphoblastoid cell line, [11] B lymphoblastoid cell line, [12,13,14,15,16,17] glioma cell lines.

Table 3.2**:** *In vitro* studies, showing the cytotoxic effects of amitriptyline, carbamazepine and gabapentin on different cell types.

Chapter: 4 Three-dimensional (3D) tissue experiments

4.1 Introduction

The effect of amitriptyline exposure on monolayer cell culture (Chapter 3), revealed high amitriptyline cytotoxicity even at low concentration and short exposure time with a measured reduction in cell count and morphological changes. However, clinical studies using amitriptyline as topical treatments did not report any tissue damage even when using high concentrations on skin and oral mucosa (Padilla *et al.*, 2000; Lynch *et al.*, 2005b). For further experiments of viability and cytotoxicity, two separate 3D tissue culture models were used to be more representative of oral mucosa and to compare with monolayer cell culture. In addition, immunohistochemistry investigations were also employed to reveal amitriptyline toxicity.

The two dimensional cell culture (2D) model is the conventional *in vitro* model used in drug discovery processes. However, the difference in cellular morphology and physiological activity, dictate the evolution of more representative *in vitro* models (Maltman and Przyborski, 2010; Edmondson *et al.*, 2014). The 3D cell culture microenvironment is thought to resemble *in vivo* tissue more accurately, where cells are arranged in a 3D spatial structure and are subject to inter-cellular and extracellular matrix interaction. All these biological interactions affect cellular, biological and physiological activity, including the response to external signalling or stimuli at the level of viability, multiplication and expression of various receptors. The development and implementation of 3D tissue cell culture in drug discovery and cytotoxicity assessments, including biocompatibility of dental materials gives more credibility to decisions based on *in vitro* data, helping to limit clinical complications, and the need for further, often costly, experiments (Klausner *et al.*, 2007; Cantòn *et al.*, 2010; Maltman and Przyborski, 2010; Edmondson *et al.*, 2014).

In this chapter, two models representing full thickness gingival tissue were used. These models, a commercial available and in–house constructed model, were both constructed of epithelial cells (keratinocytes) and gingival fibroblasts on collagen scaffolds (Section 2.5). The two types of 3D model used were: commercial MatTek model, which is constructed from primary human gingival cells, keratinocytes and fibroblast cells (EpiOral™ and EpiGingival ™, MatTek Corporation, USA); the in-house constructed model constructed from keratinocytes cell line (FNB6 cell line from human oral dysplasia) and primary gingival fibroblasts. Both models were constructed on collagen scaffolds in tissue culture inserts and transwell plates respectively, (ethical approval number 09/H1308/66), (Klausner *et al.*, 2007; Colley *et al.*, 2011), (Section 2.5).

In this chapter, alamarBlue® was used to investigate viability using short and long exposure time of amitriptyline. Repeated exposure time points were used to simulate repeated clinical application as well as periods of recovery with the aim to investigate the possibility of reversible damage that could occur with chronic use.

The cytotoxicity effect was investigated using the Lactate dehydrogenase assay (LDH); (CYTOTOX96®, Promega, UK), using the same time points as those used in the viability study (using conditioned culture media from both MatTek and in-house constructed models). In this chapter, two types of anti-caspase 3 antibodies were used to investigate the apoptotic effect of amitriptyline in both 3D models and at all time points as previous studies reported an apoptotic effect of amitriptyline on cell culture models through activation of the caspase 3 pathway (Lirk *et al.*, 2006a).

Employing 3D tissue with air–liquid interphase culture environment was used to simulate and measure actual drug absorption. The percentage of amitriptyline passage through epithelial tissue to the expected target nerve endings was investigated using both chemical and immunofluorescence studies, through HPLC and immunofluorescent microscope (Section 2.8.1 & 2.12).

4.2 Viability of 3D models

The alamarBlue® assay (Section 2.6) was used to investigate the changes in 3D tissues viability after amitriptyline exposure. The exposure time points were limited to the expected clinical course and the amitriptyline concentration used was the concentration found in HPLC results presented in Chapter 3 (Section 3.4), (226 µM).

4.2.1 Viability of MatTek 3D tissue

Viability was measured after the commercially available MatTek model was exposed to amitriptyline at a concentration of 226 µM applied to the apical service of the tissue inserts. Four time points were measured in the MatTek model: 30 min, 24 h and repeated exposure for three days: where amitriptyline was applied twice a day (B.I.D.) and three times a day (T.I.D.).

The results showed a reduction in viability after the two repeated exposure time points (B.I.D. and T.I.D.); however, this change in viability was only significant with B.I.D. exposure time point ($p<0.05$). The 30 min and 24 h exposure did not show any reduction in viability, Figure 4.1.

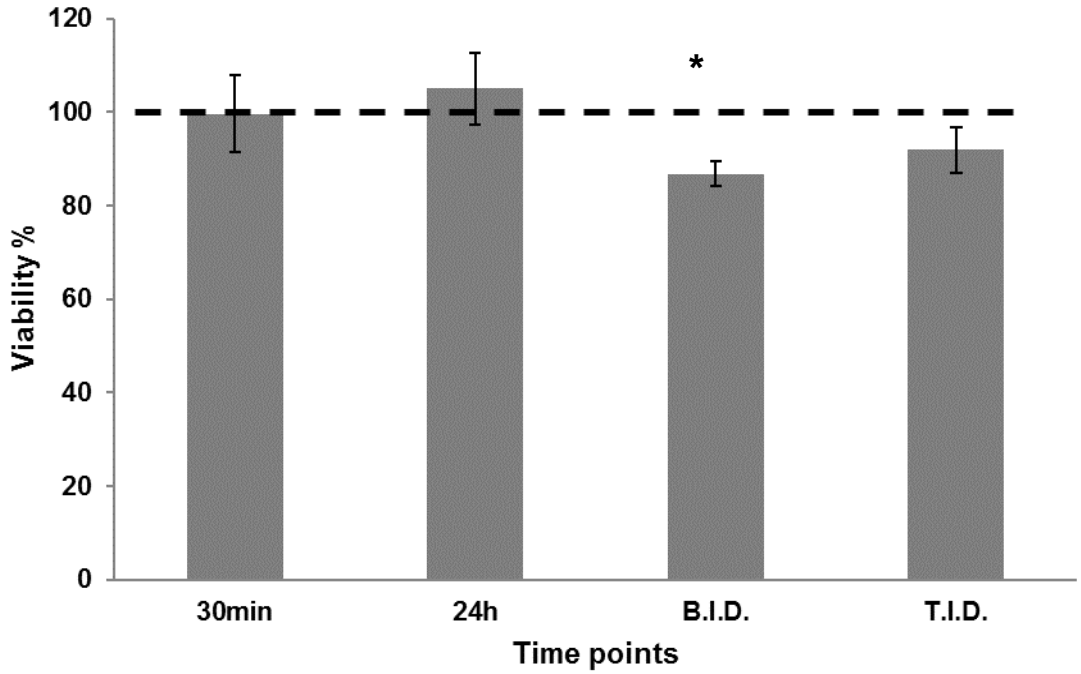


Figure 4.1: Cellular viability of the MatTek 3D tissue models exposed to amitriptyline (226 µM) for 30 min, 24 h, twice a day (B.I.D.) and three times a day (T.I.D.) for three days. Mean values ± SE (n=3) are shown for each experiment compared with untreated control tissue shown as 100 % viable (black dotted line) by one-way ANOVA followed by Bonferroni's post hoc test, * $p<0.05$.

4.2.2 Viability of in-house constructed 3D tissue

a- Viability of cells were used to construct the in-house 3D model

The median lethal dose (LD_{50}) of amitriptyline was investigated in the cell line (FNB6) and primary cells (primary human gingival fibroblast cells, passage 9) used to construct the in-house 3D models. The experiments were carried out separately from the tissue used to construct, at short and long time points (30 min, 24 h) of amitriptyline exposure.

The results of amitriptyline exposure on FNB6 were investigated using a MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), (Figure 4.2). In gingival fibroblasts, LD_{50} values were investigated using alamarBlue® at 30 min and 24 h time points, (Figure 4.3). The results of amitriptyline exposure showed high cytotoxicity of amitriptyline even at low concentrations. The calculation of LD_{50} was made without subtracting the media absorption to draw the curve above zero. The LD_{50} values for both cell lines are shown in Table 4.1.

Although two different methods were used to calculate the median lethal dose of amitriptyline (MTT, alamarBlue®), changed due to convenience in laboratories (Sheffield and Newcastle Universities), the results are almost identical, Table 4.1.

	Cell types	30 min (µM)	24 h (µM)
A	FNB6 cell line (using MTT assay)	204	50
B	Fibroblast cell (using alamarBlue®)	204	52.4

Table 4.1: Median Lethal dose values for amitriptyline were investigated in: (A) FNB6 cell line at 30 min and 24 h exposure time points using MTT assay. (B) Human gingival fibroblast cells at 30 min and 24 h exposure time points using alamarBlue® assay, n=6.

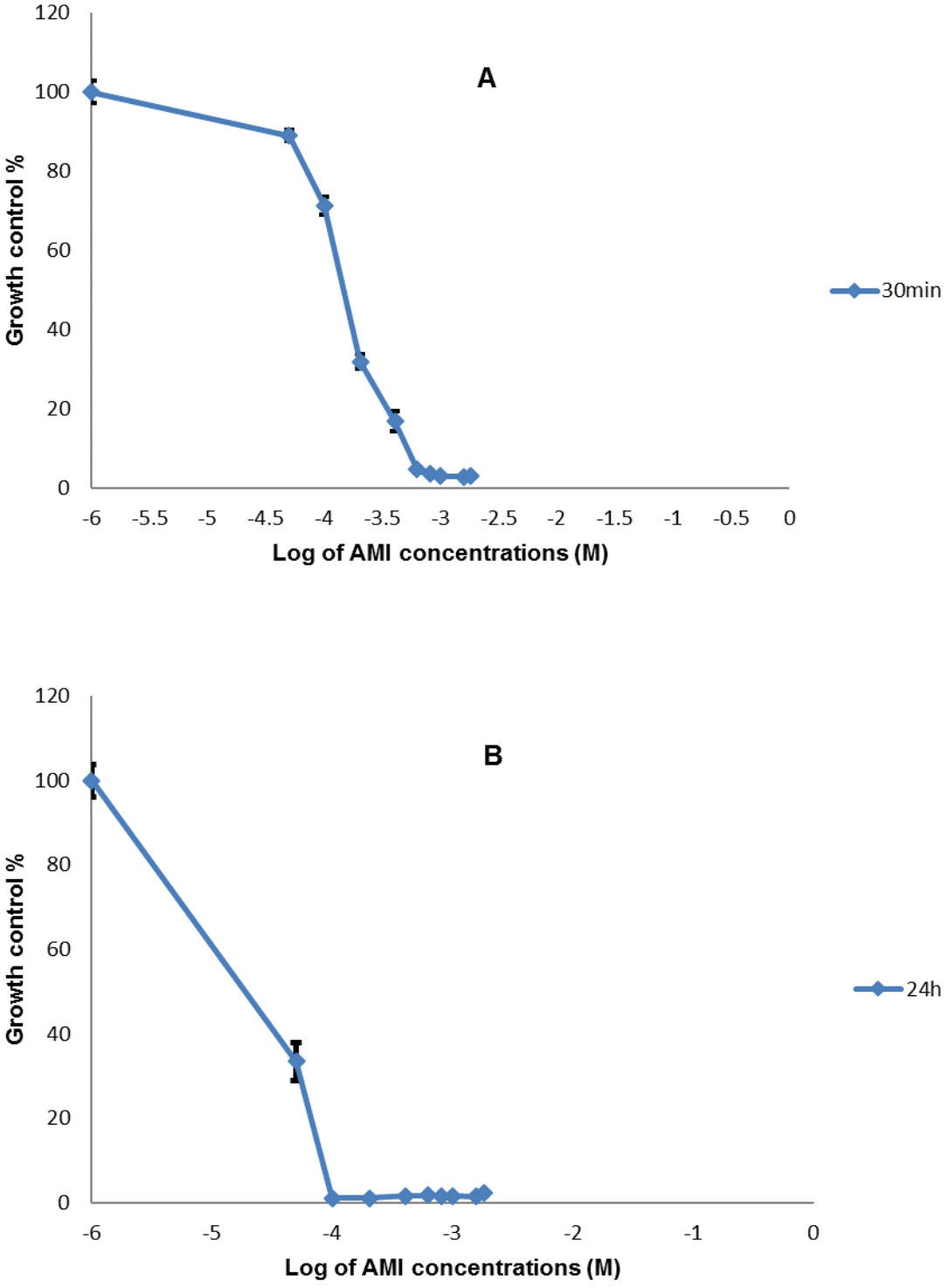
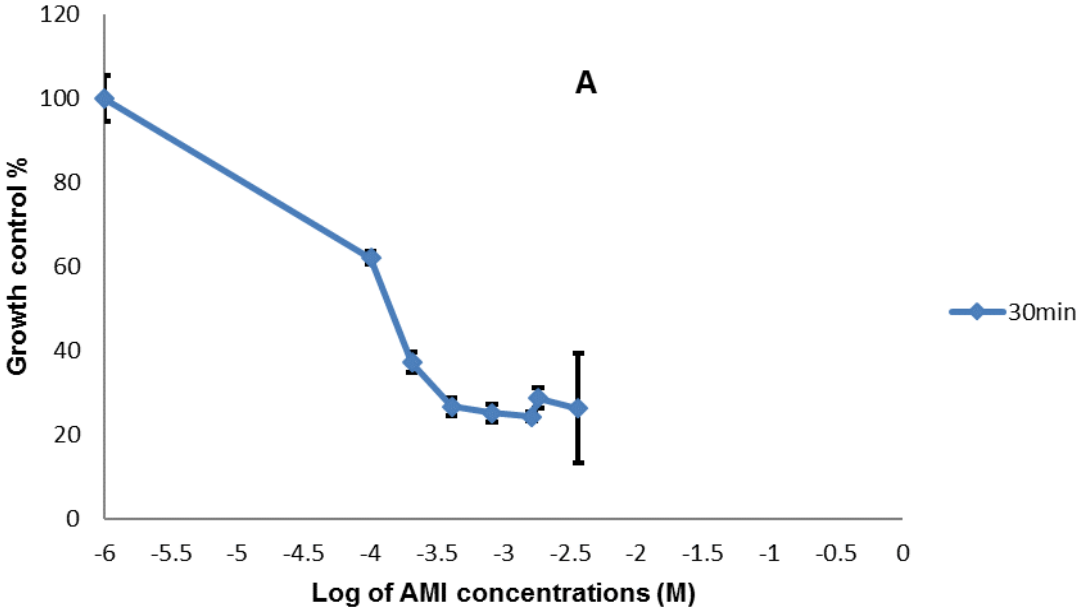


Figure 4.2: Amitriptyline (AMI) median lethal dose values were calculated using MTT assay in FNB6 cell line after (A) 30 min and (B) 24 h exposure time points. Mean values ± SE (n=6) are shown for each experiment.



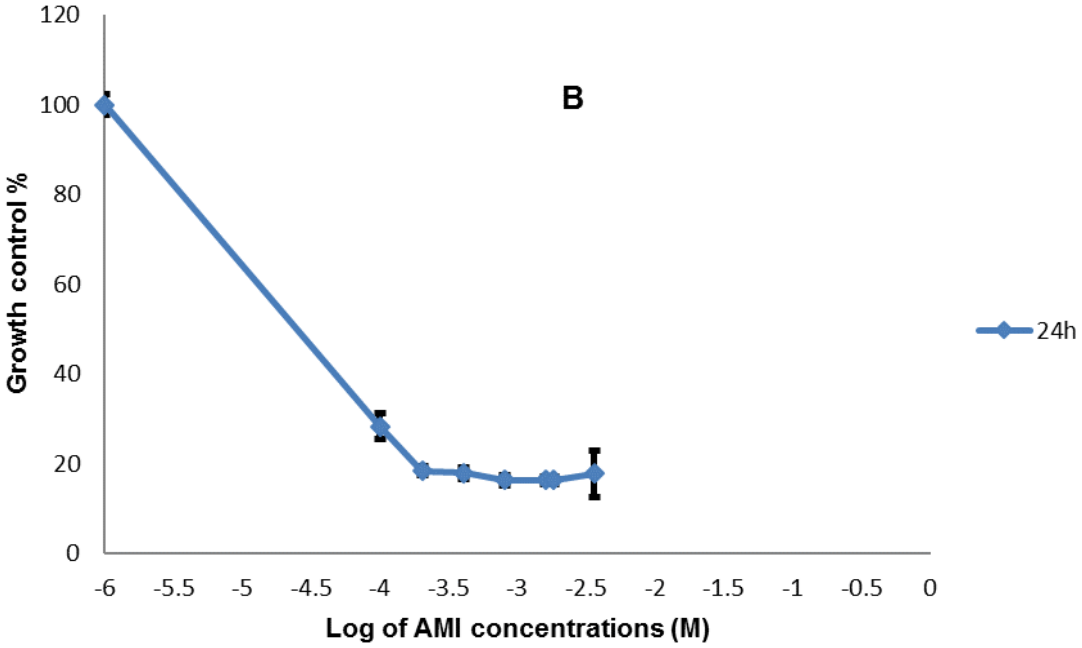


Figure 4.3: Amitriptyline (AMI) median lethal dose values were calculated using alamarBlue® assay in human gingival fibroblast cells after (A) 30 min and (B) 24 h exposure time points. Mean values ± SE (n=6) are shown for each experiment.

b- Viability results of the in-house constructed 3D tissue

The in-house constructed 3D models were exposed to amitriptyline at 30 min and 24 h time points only. The repeated exposure time points (B.I.D. and T.I.D.) were not employed in this model as the 3D model was not robust for repeated exposure experiments, in addition to the restriction of the laboratory procedure at Sheffield University, UK.

The exposure of the in-house constructed model to amitriptyline at 30 min and 24 h, showed similar results to that found in the commercial MatTek model experiments, Figure 4.4. There was a reduction after 24 h amitriptyline exposure, but both time points differences were non-significant comparing with control untreated tissues ($p>0.05$).

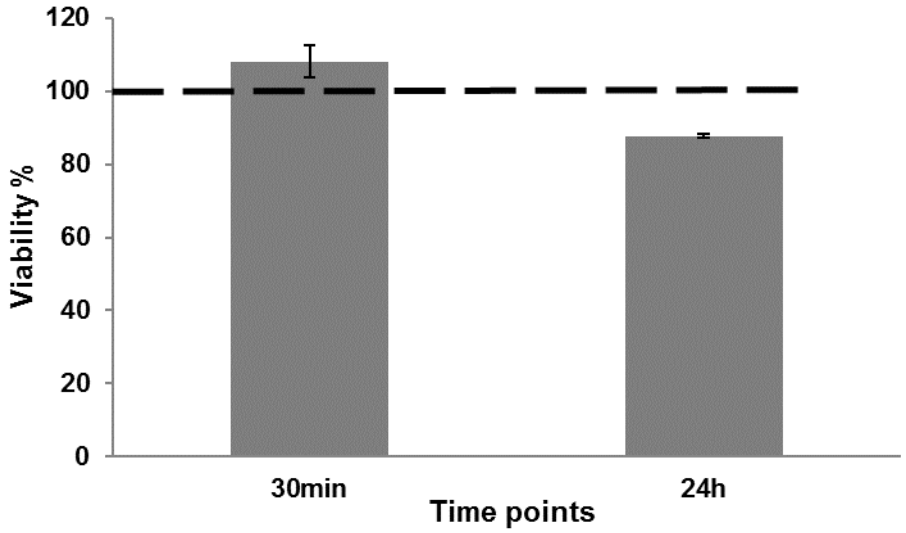


Figure 4.4: Cellular viability of the in-house constructed 3D tissue models was measured using alamarBlue®, exposed to amitriptyline (226 µM) at 30 min, 24 h time points. Mean values ± SE (n=3) are shown for each experiment compared with untreated control cells by parametric independent t- test, *p>0.05.

4.3 Cytotoxicity results

The cytotoxicity of amitriptyline was also investigated using LDH assay on conditioned media (media bathing tissue models after treatment) collected after amitriptyline exposure. The time points used for cytotoxicity assay were the same as in the viability experiment (30 min, 24 h, B.I.D. and T.I.D.).

4.3.1 Cytotoxicity of amitriptyline in the MatTek model

Conditioned media were collected after each exposure to amitriptyline in the 3D tissue model. For repeated exposure, the culture media were collected at the end of experiment day and was submitted to LDH assay to investigate also if repeated exposure affects the cytotoxicity profile differently to single exposure.

Absorbance was measured at 490 nm, a higher reading indicates an increase in LDH activity and cell death. The only significant difference observed was at 24 h exposure time point where increase in LDH level was measured in both control and treated tissue models, (Figure 4.5).

4.3.2 Cytotoxicity of amitriptyline in the in-house constructed model

The in-house constructed model was exposed to amitriptyline for 30 min and 24 h and showed significant level of LDH at both time points comparing with control tissue samples ($p<0.05$). The higher change was after 24 h of exposure as shown in Figure 4.6.

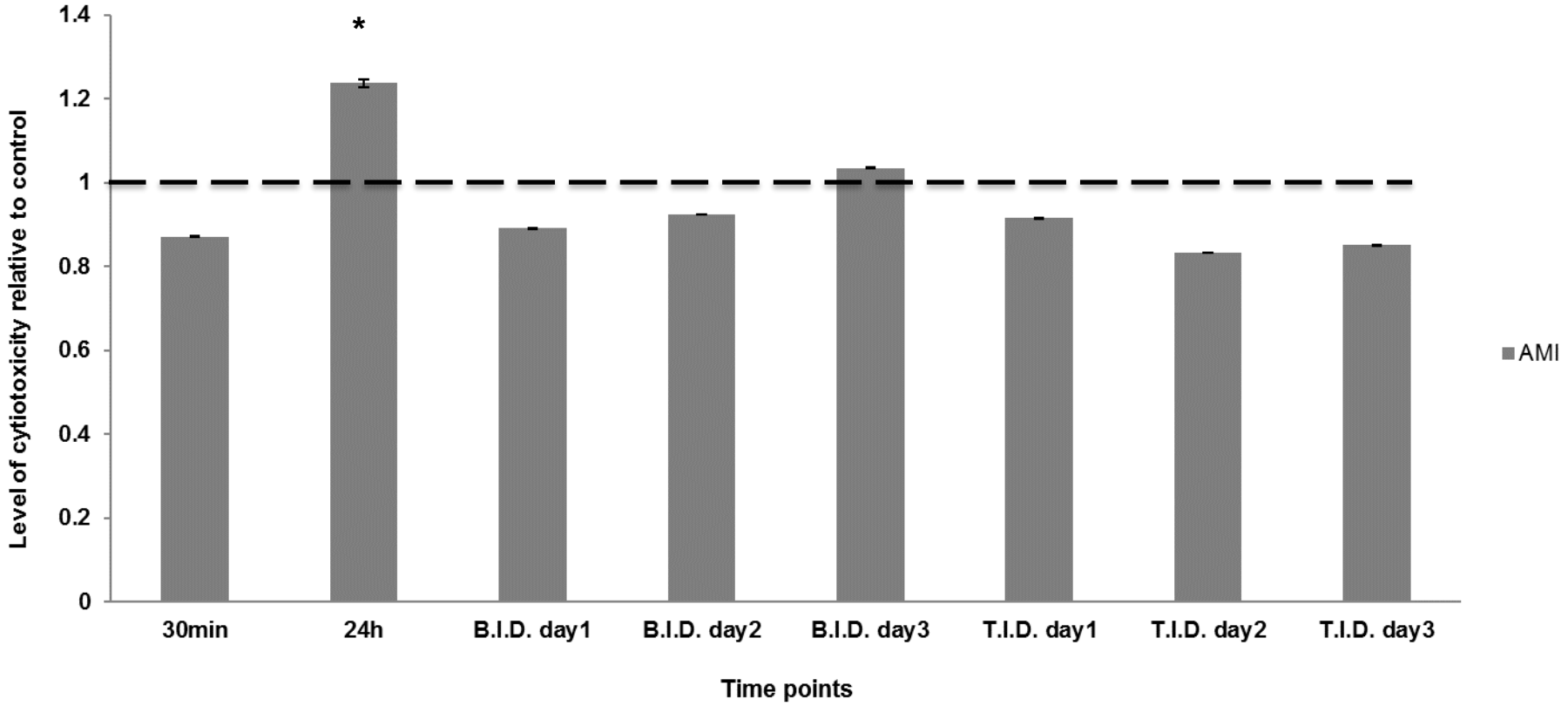


Figure 4.5: Cytotoxicity results of the MatTek 3D tissue models were exposed to amitriptyline (AMI), (226 µM) for 30 min, 24 h, twice a day (B.I.D.) and there times a day (T.I.D.) for three days. Mean values ± SE (n=3) are shown for each experiment after were normalised to control results. Compared with control results (black dotted line) at the same time points by parametric independent t-test, * $p<0.05$.

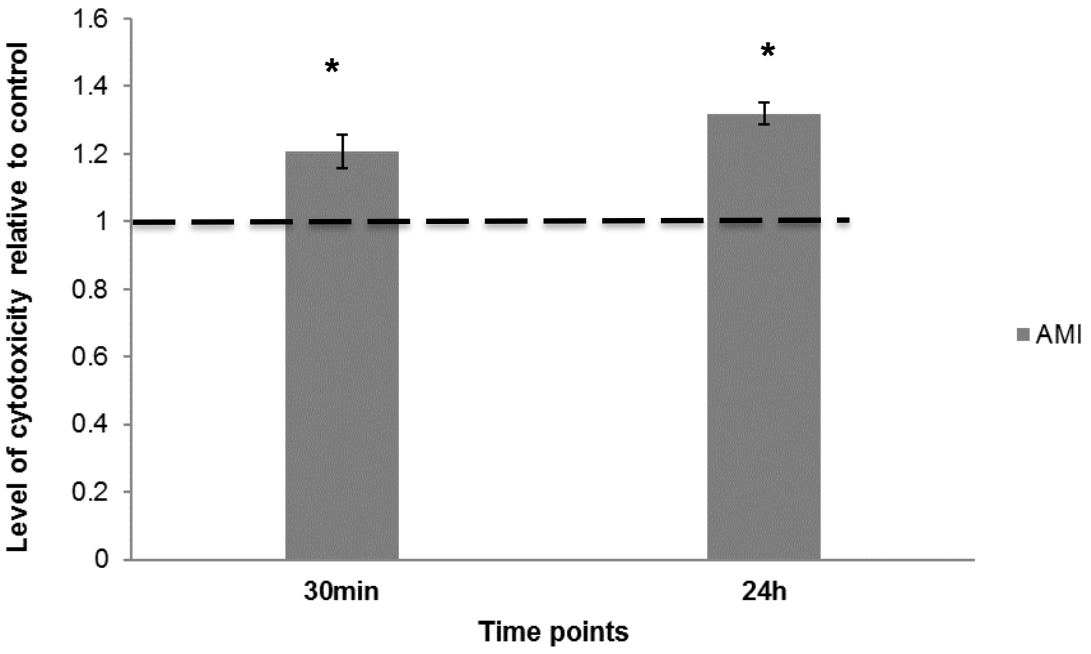


Figure 4.6: Cytotoxicity results of the in-house constructed 3D tissue models were exposed to amitriptyline (AMI), (226 µM) at 30 min, 24 h time points. Mean values ± SE (n=3) are shown for each experiment after normalised to control results at the same time points. Compared with untreated control cells (black dotted line) by parametric independent t-test, *p<0.05.

4.4 H&E immunohistochemistry study to investigate tissue integrity

To explore the amitriptyline effect on 3D models cellular and tissue integrity, H&E staining was employed. After viability experiments were conducted, the 3D tissues were fixed using 10 % formalin and tissue were embedded in wax before slides were prepared. Sections were stained with H&E and visualized by light microscope. The slides were examined in blind way regarding the exposure time points, groups and the tissue types.

4.4.1 Tissue integrity of MatTek model by H&E

After 30 min and 24 h exposure of amitriptyline, careful examination of the H&E slides at 20 x and 40 x magnification, did not reveal any visual changes within tissues including keratin and cellular layers comparing with control tissues, 30 min and 24 h exposure time points are shown in Figure 4.7 and Figure 4.8 respectively. Three slides for each time points were examined for each time point.

Repeated exposure to amitriptyline for 3 days with B.I.D. and T.I.D. exposure time points, did not produce major changes, however, slight rounding and shrinkage in the cellular layer was observed in the case of treatment group, which was more pronounced with T.I.D. exposure.

B.I.D. and T.I.D. exposure time points are shown in Figure 4.9 and Figure 4.10 respectively.

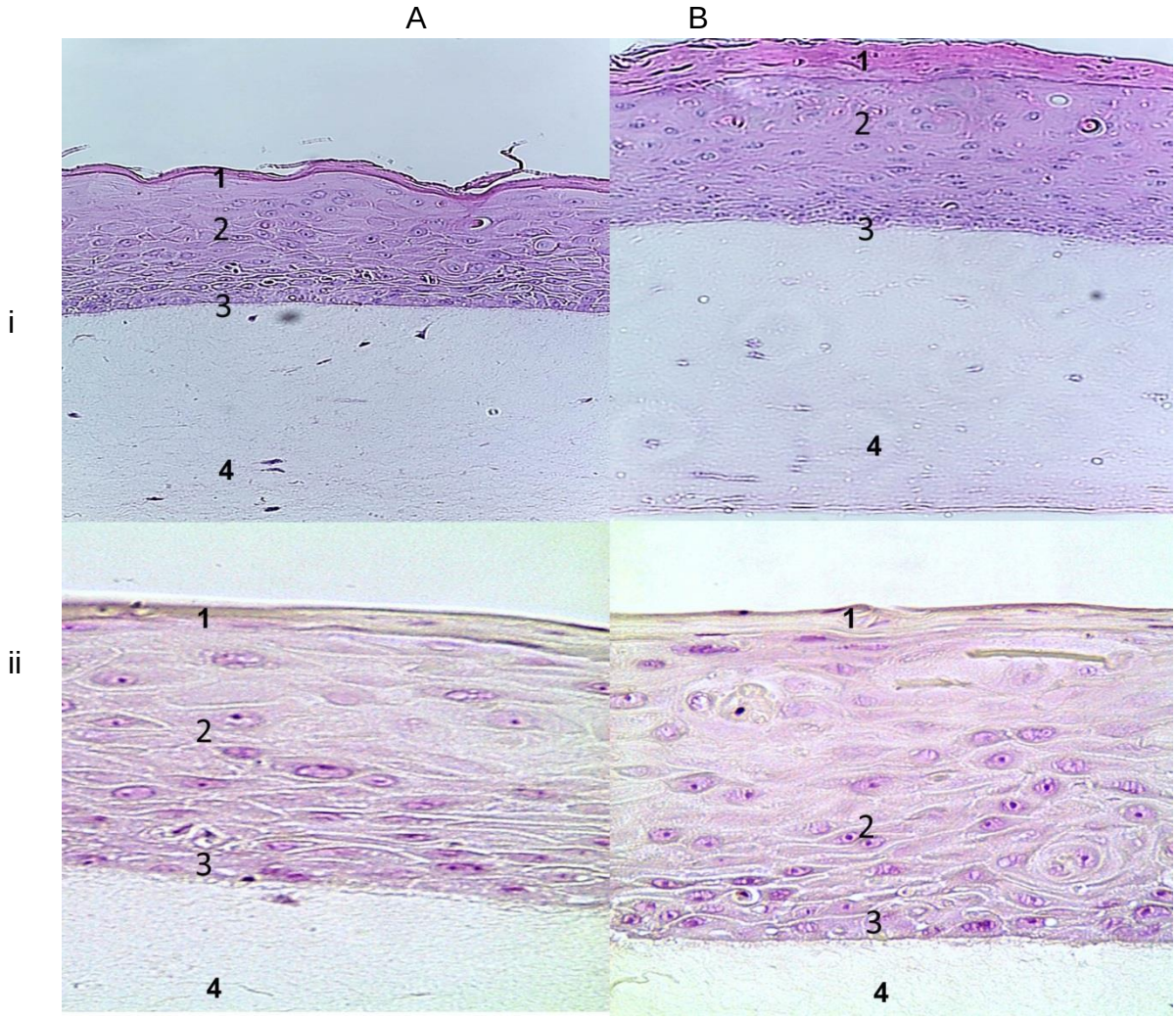


Figure 4.7: Representative H&E stained slides from MatTek tissue after 30 min exposure to amitriptyline (226 µM), n=3. A: control tissue images at (i) 20 X and (ii) 40 X, B: amitriptyline exposed tissue images at (i) 20 X and (ii) 40 X. Layers indicated: 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer.

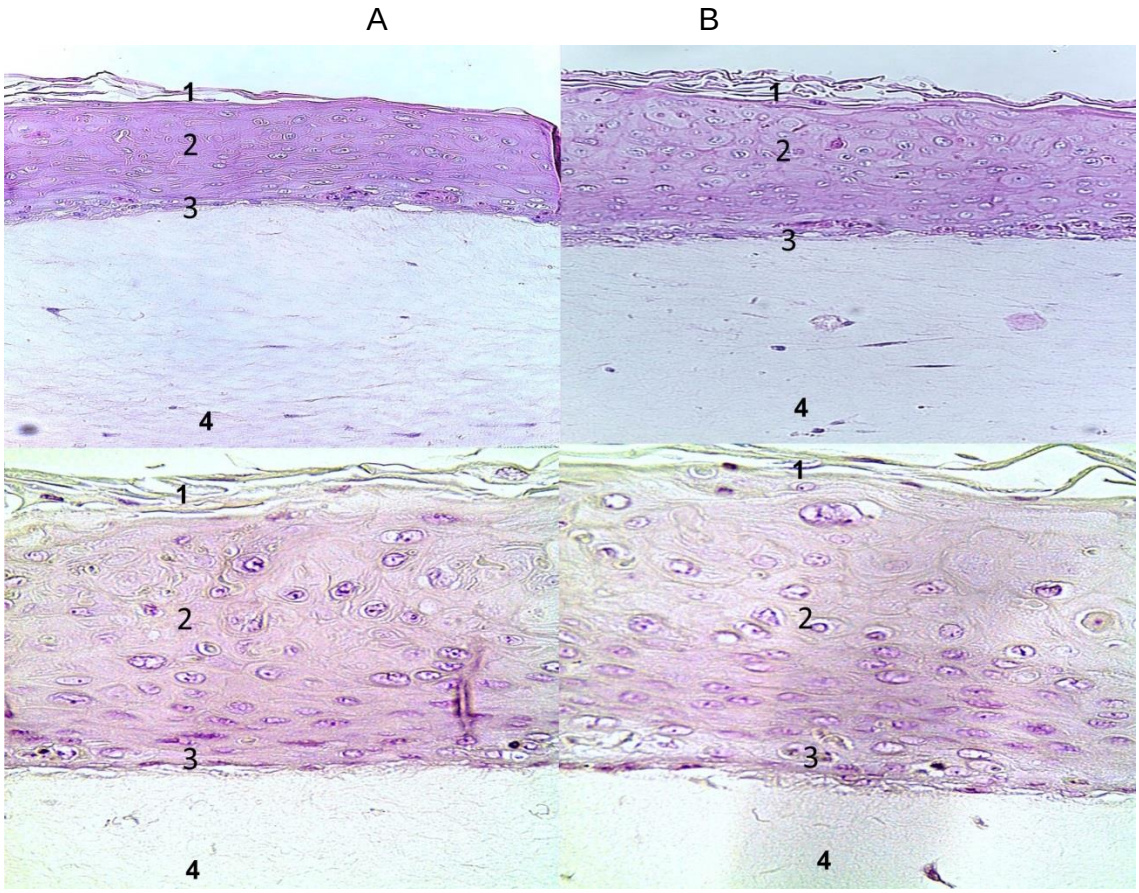


Figure 4.8: Representative H&E stained slides from MatTek tissue after 24 h exposure to amitriptyline (226 µM), n=3. A: control tissue images at (i) 20 X and (ii) 40 X, B: amitriptyline exposed tissue at (i) 20 X and (ii) 40 X. Layers indicated: 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

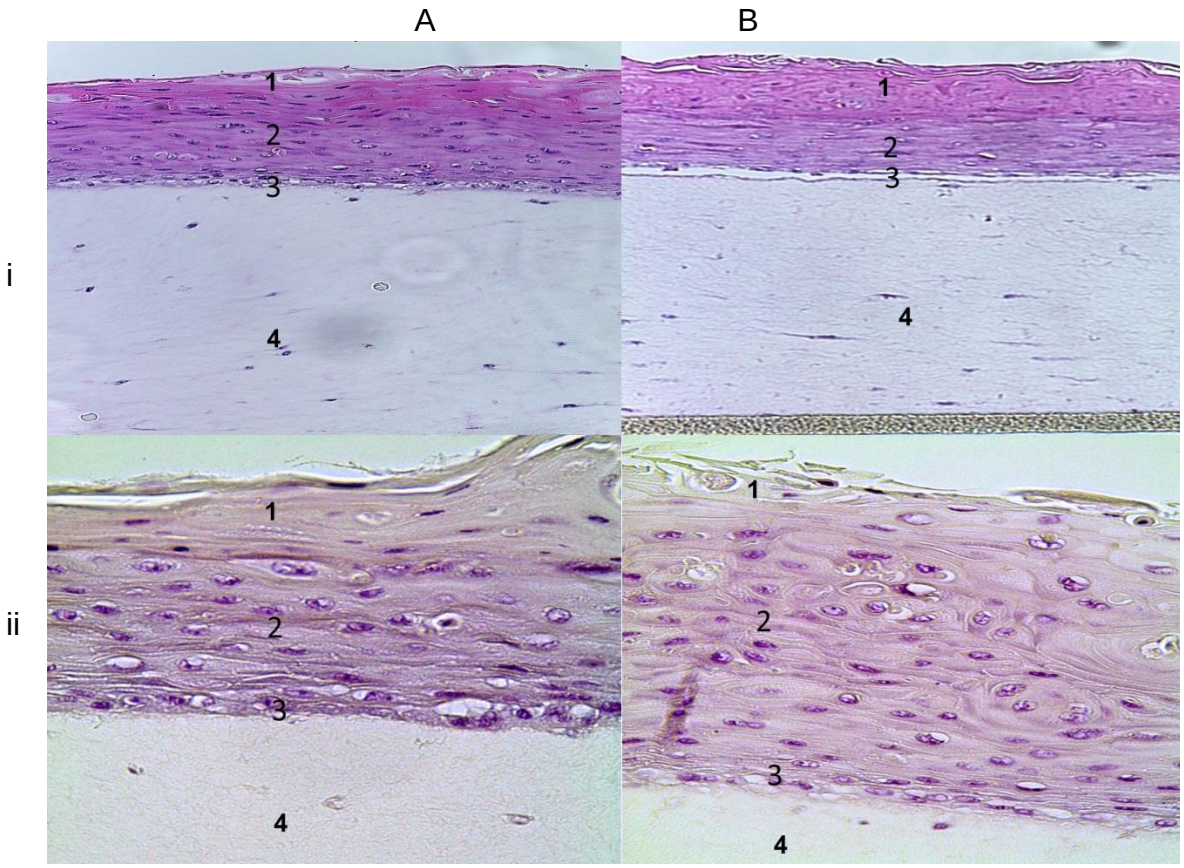


Figure 4.9: Representative H&E stained slides for MatTek tissue after twice a day (B.I.D.) exposure for three days to amitriptyline (226 µM), n=3. A: control tissue images at (i) 20 X and (ii) 40 X, B: amitriptyline exposed tissue at (i) 20 X and (ii) 40 X. Layers indicated: 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer.

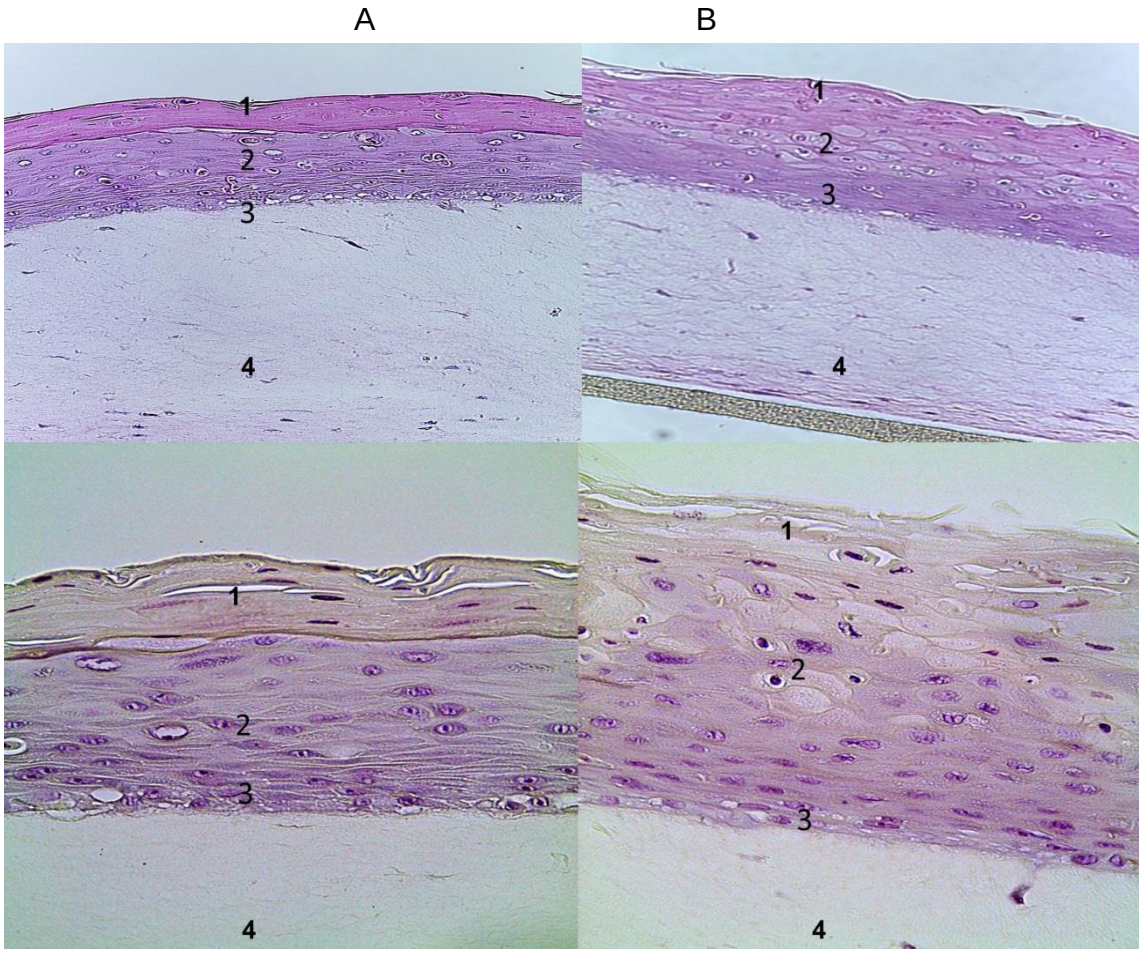


Figure 4.10: Representative H&E stained slides for MatTek tissue after three times a day (T.I.D.) exposure for three days to amitriptyline (226 µM), n=3. A: control tissue images at (i) 20 X and (ii) 40 X, B: amitriptyline exposed tissue at (i) 20 X and (ii) 40 X. Layers indicated: 1: keratin layer, 2: cellular layer, 3: basal layer, 4: submucosa layer collagen layer).

4.4.2 In-house Constructed 3D tissue results

The in-house constructed model was exposed to amitriptyline at 30 min and 24 h. The results of H&E experiment for both time points expressed slight changes of cells including rounding and shrinkage between treated tissues compared with control untreated tissues at the same time points investigated.

Figure 4.11 and Figure 4.12 are shown 30 min and 24 h results respectively.

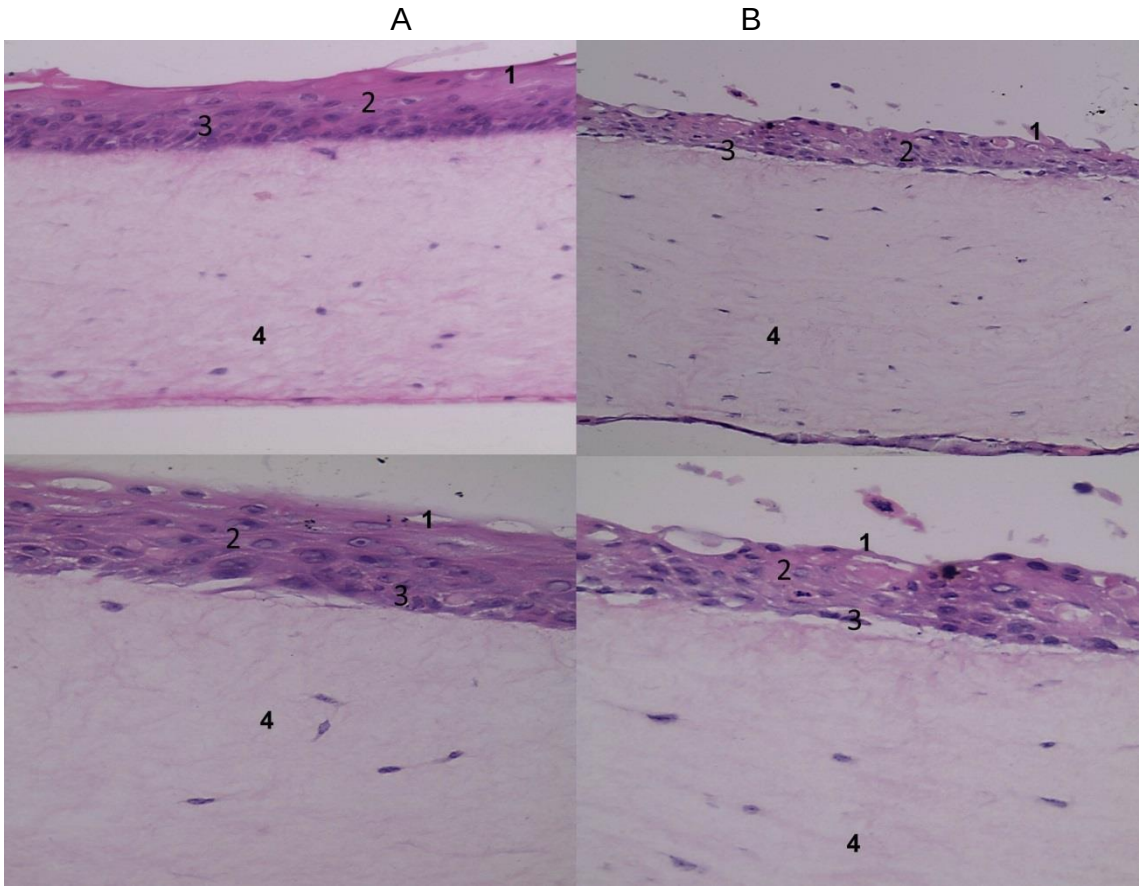


Figure 4.11: Representative H&E stained slides for the in-house constructed tissue after 30 min exposure to amitriptyline (226 µM), n=1. A: control tissue images at (i) 20 X and (ii) 40 X, B: amitriptyline exposed tissue at (i) 20 X and (ii) 40 X. Layers indicated: 1: keratin layer, 2: cellular layer, 3: basal layer, 4: submucosa layer collagen layer.

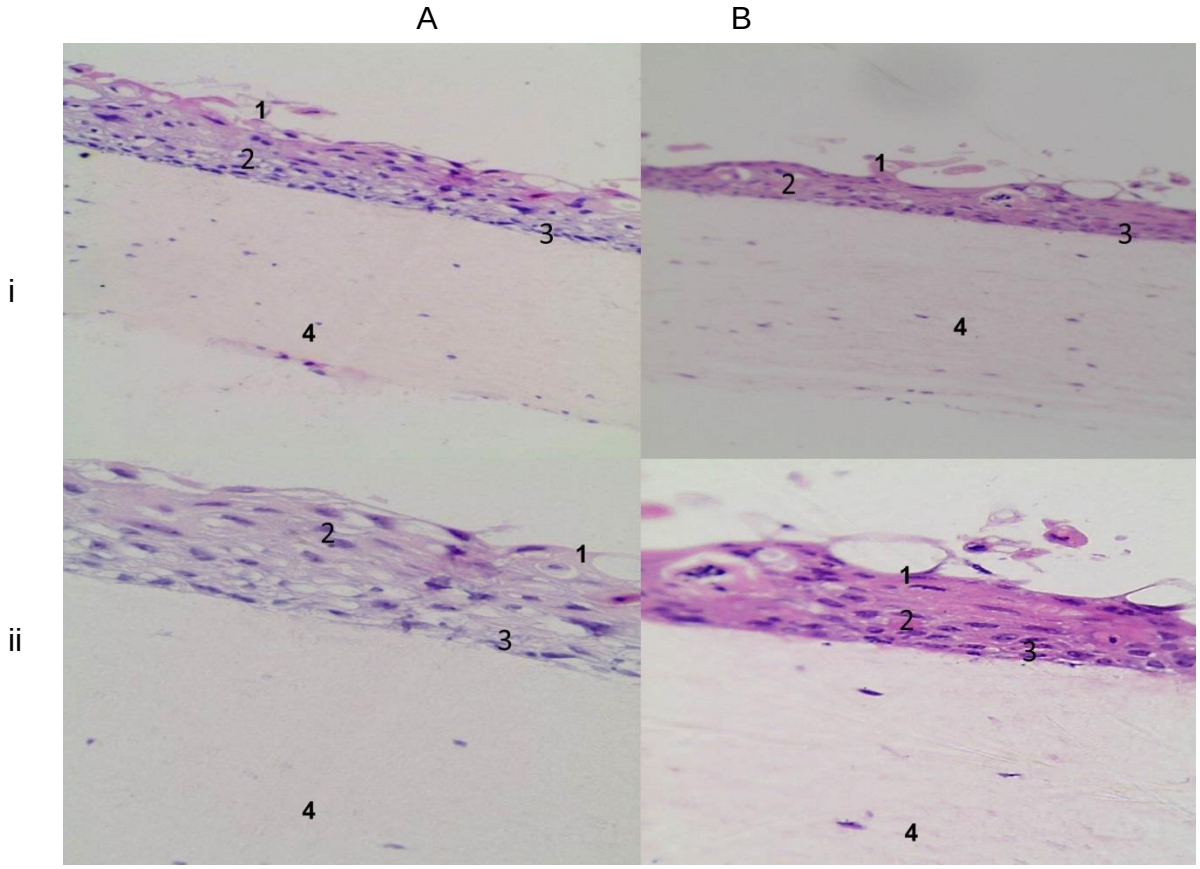


Figure 4.12: Representative H&E stained slides for the in-house constructed tissue after 24 h exposure to amitriptyline (226 µM), n=1. A: control tissue images at (i) 20 X and (ii) 40 X, B: amitriptyline exposed tissue at (i) 20 X and (ii) 40 X. (1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

4.5 Investigation of the apoptotic effect of amitriptyline in 3D model

The cytotoxic effect of amitriptyline is expected to be associated with apoptotic activity even at low concentrations, to investigate this probability, two types of apoptosis antibody were used to investigate cellular apoptosis throughout the 3D tissue model in response to amitriptyline exposure: Menapath polyclonal Caspase 3 antibody (Biocare LLC, California, USA) and a rabbit monoclonal caspase 3 antibody from R&D systems 269518 (R&D Systems, Inc., USA).

4.5.1 Caspase 3 level in MatTek 3D model

Immunohistochemistry results are presented as microscopic images for all time points, with usual counting of positively expressed anti-caspase 3 antibody results from 9 images representing 3 tissues for each group, compared with the same numbers of images and tissues from control slides results. The counting was carried out using light microscope and 50 % of results were calibrated by another researcher independently.

a: Menapath polyclonal Caspase 3 in the MatTek model:

Short time exposure of 30 min, showed less apoptotic cells in both control and treatment groups when compared with the other exposure time points. The counting of expressed antibody, apoptotic cells, was not significant when compared with control results ($p>0.05$), (Figure 4.13, Figure 4.17).

Long time exposure time point (24 h), was associated with a significant difference of expressed apoptotic activity when compared with control ($p<0.05$). The slides images present with high expression of anti-caspase 3 antibody positive results (

Figure 4.14, Figure 4.17).

Repeated exposure time points (B.I.D., T.I.D.) were associated with a higher numbers of apoptotic cells when compared with controls at the same time points. The counting of expressed antibody was significant at both time points

when compared with control ($p<0.05$); Figure 4.15, shows B.I.D. time point results and Figure 4.16 shows T.I.D. time points results. Results for all apoptotic counting experiments are amalgamated in Figure 4.17.

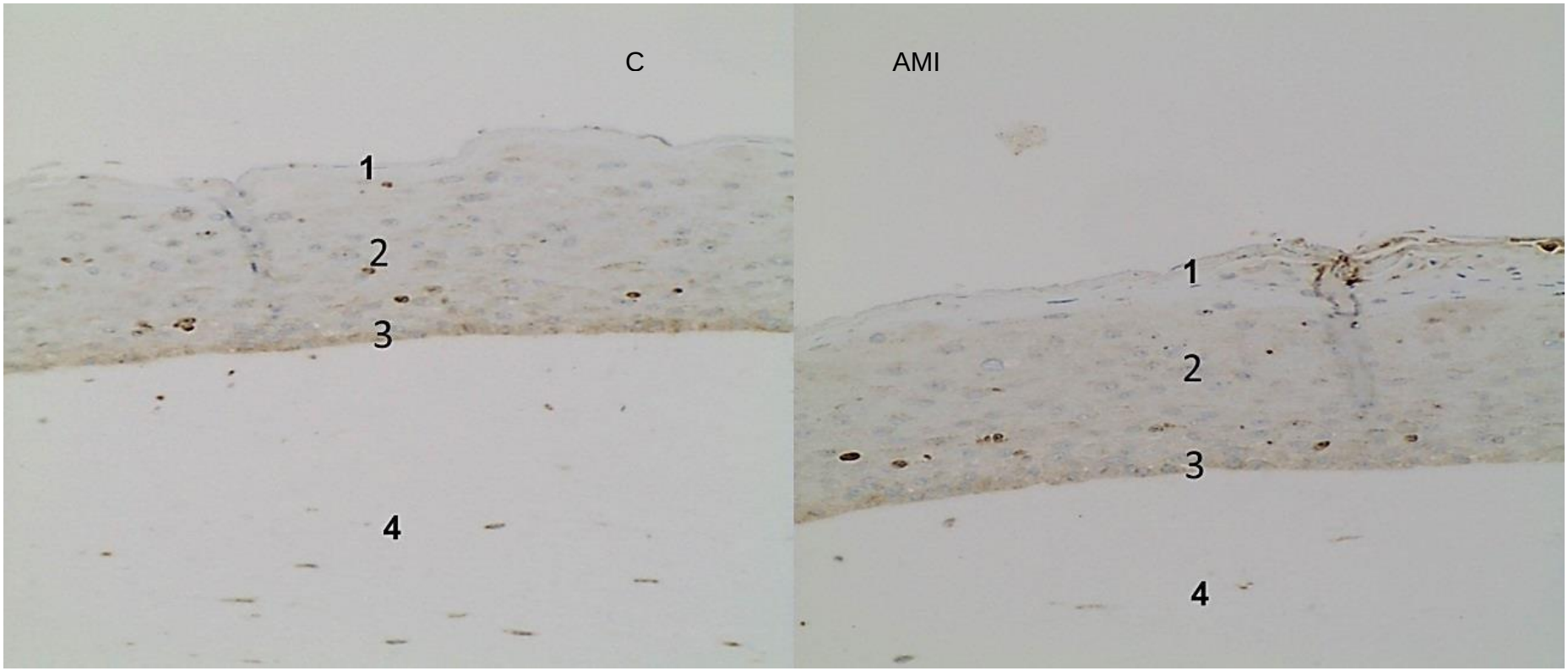


Figure 4.13: Menapath polyclonal Caspase 3 immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) for 30 min, n=3. Images shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

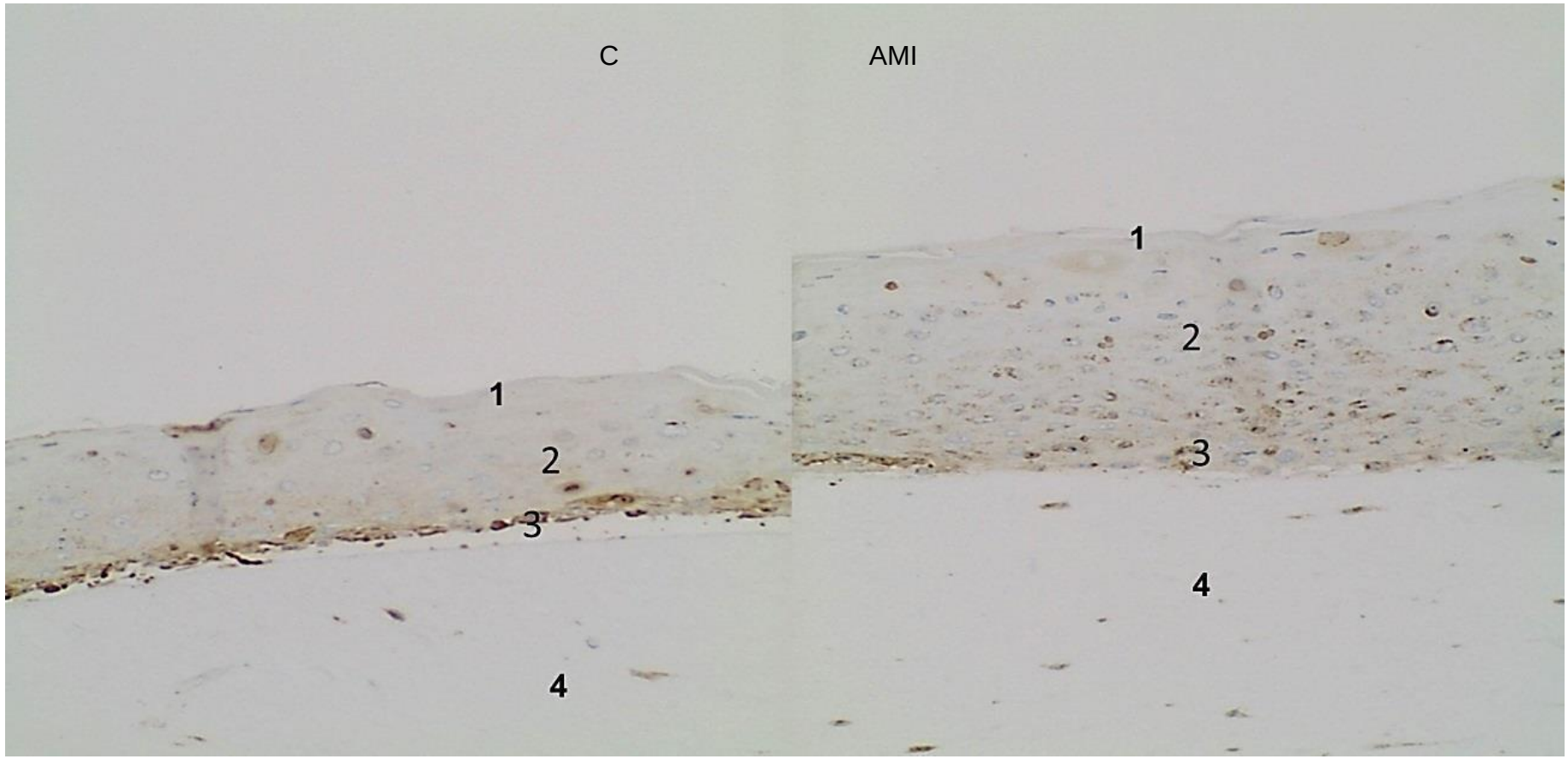


Figure 4.14: Menapath polyclonal Caspase 3 immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) for 24 h, n=3. Images shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

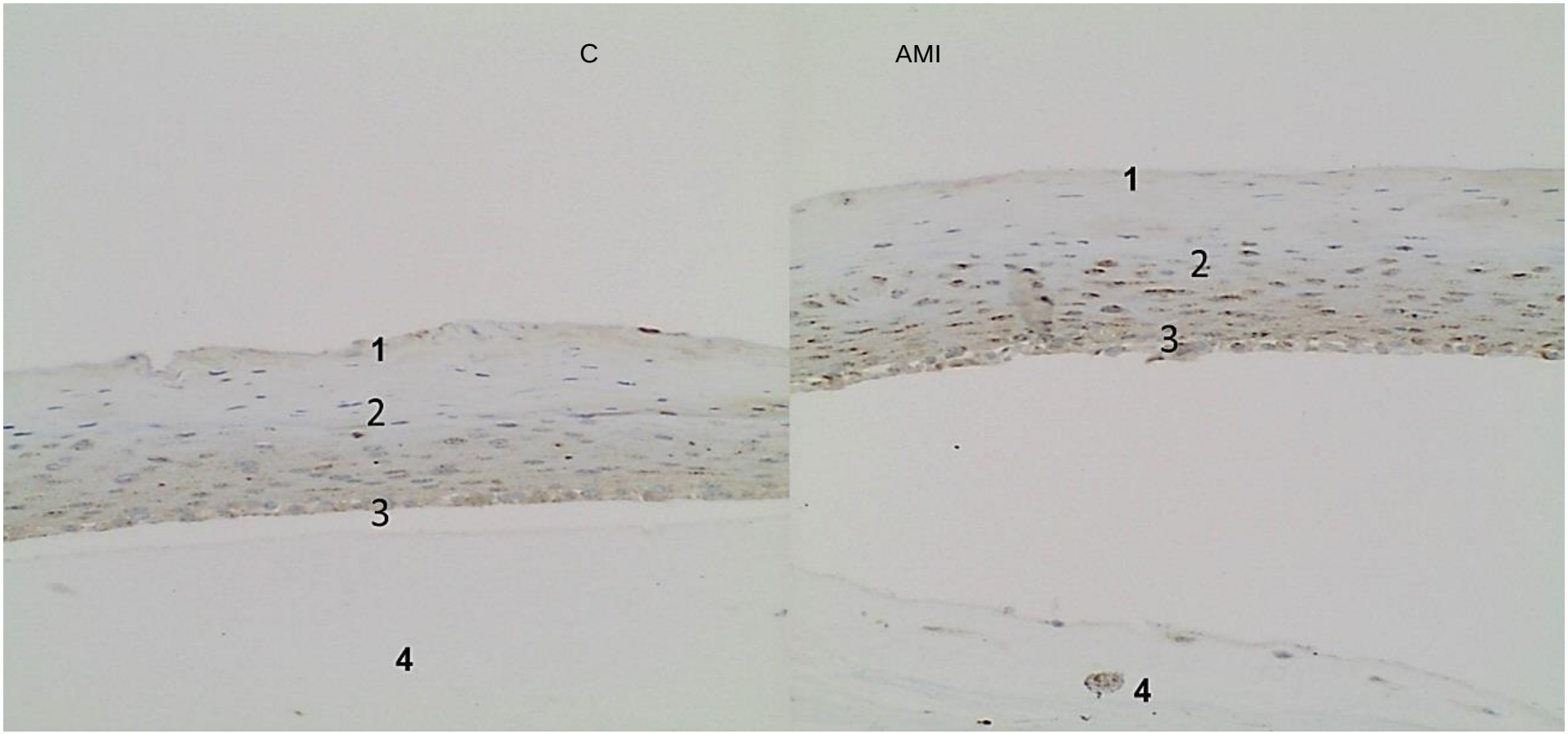


Figure 4.15: Menapath polyclonal Caspase 3 immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) twice a day (B.I.D.) for three days, n=3. Images shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

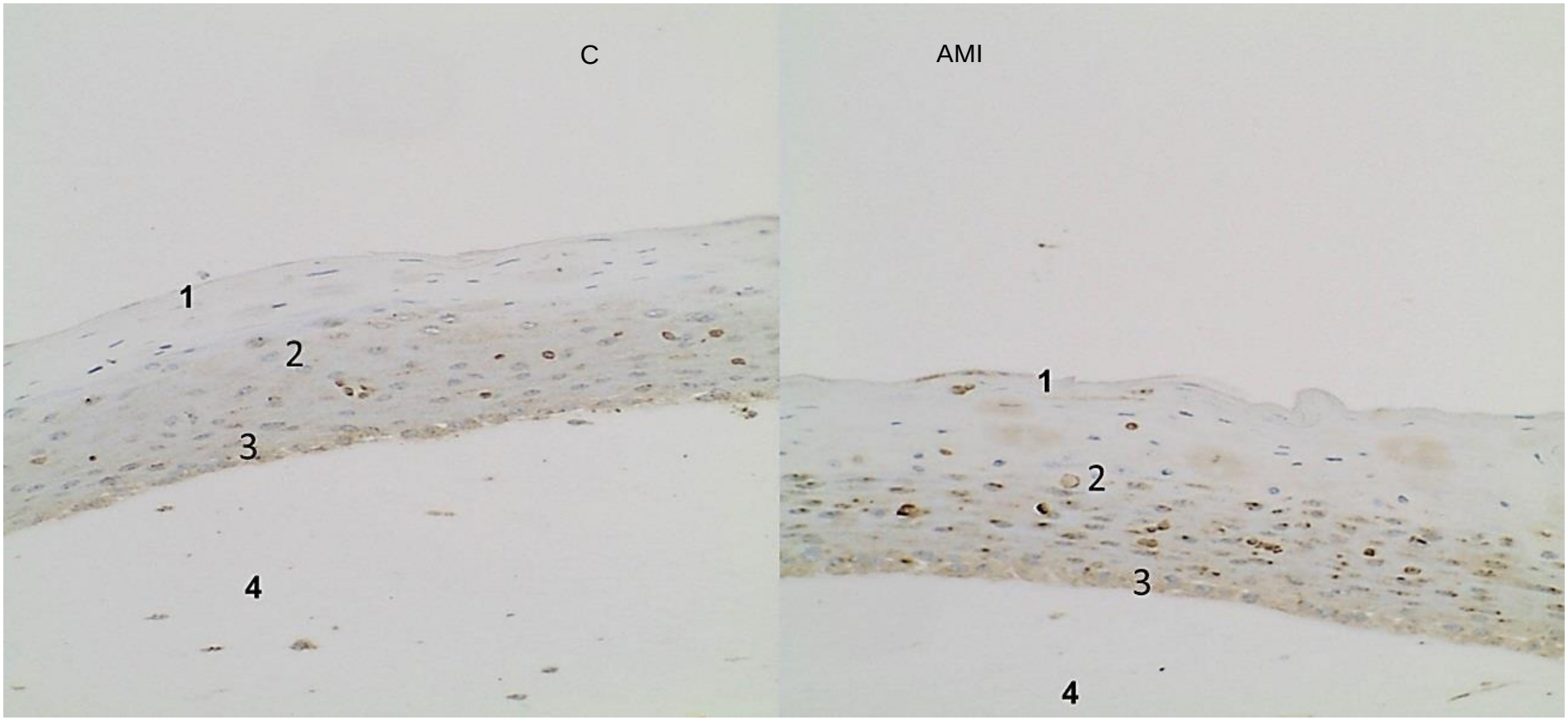


Figure 4.16: Menapath polyclonal Caspase 3 immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) Three times a day (T.I.D.) for three days, n=3. Images shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

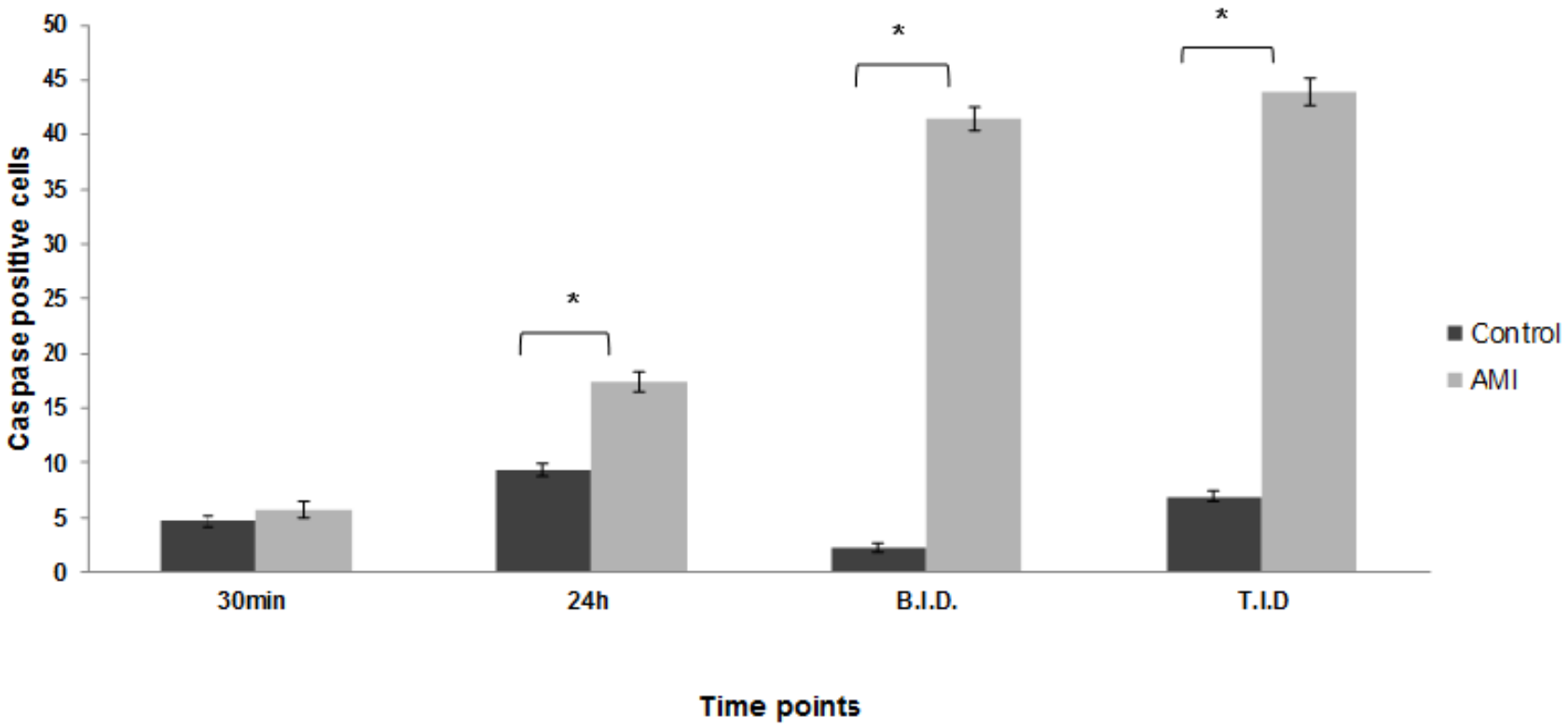


Figure 4.17: The results of Menapath polyclonal Caspase 3 immunohistochemistry study of the MatTek tissue was exposed to amitriptyline (AMI), (226 µM) at 30 min, 24 h, B.I.D. and T.I.D., the results are mean ± SE (n=3 tissues, n=9 images analysed and presented), the results were compared with control using parametric and nonparametric independent t-test, *p<0.05.

b: Rabbit monoclonal from R&D systems in the MatTek model:

The second antibody investigation showed less overall apoptotic expression in both treated and control tissues at all time points. Significant differences between treated and control tissues were similar to those seen in the Menapath polyclonal Caspase 3 experiment.

Short exposure time (30 min) showed non-significant difference in numbers of apoptotic cells between control and treated cells, ($p>0.05$), (Figure 4.18, Figure 4.22). Long exposure time point (24 h) was associated with a higher number of apoptotic cells even more than the repeated exposure and the results were significant comparing with control ($p<0.05$) (Figure 4.19, Figure 4.22).

Repeated exposure time points (B.I.D., T.I.D.) were associated with higher numbers of apoptosis and significantly different when were compared with control tissue results at both time points (B.I.D., T.I.D.), (B.I.D. results: Figure 4.20, Figure 4.22 ; T.I.D. results : Figure 4.21, Figure 4.22).

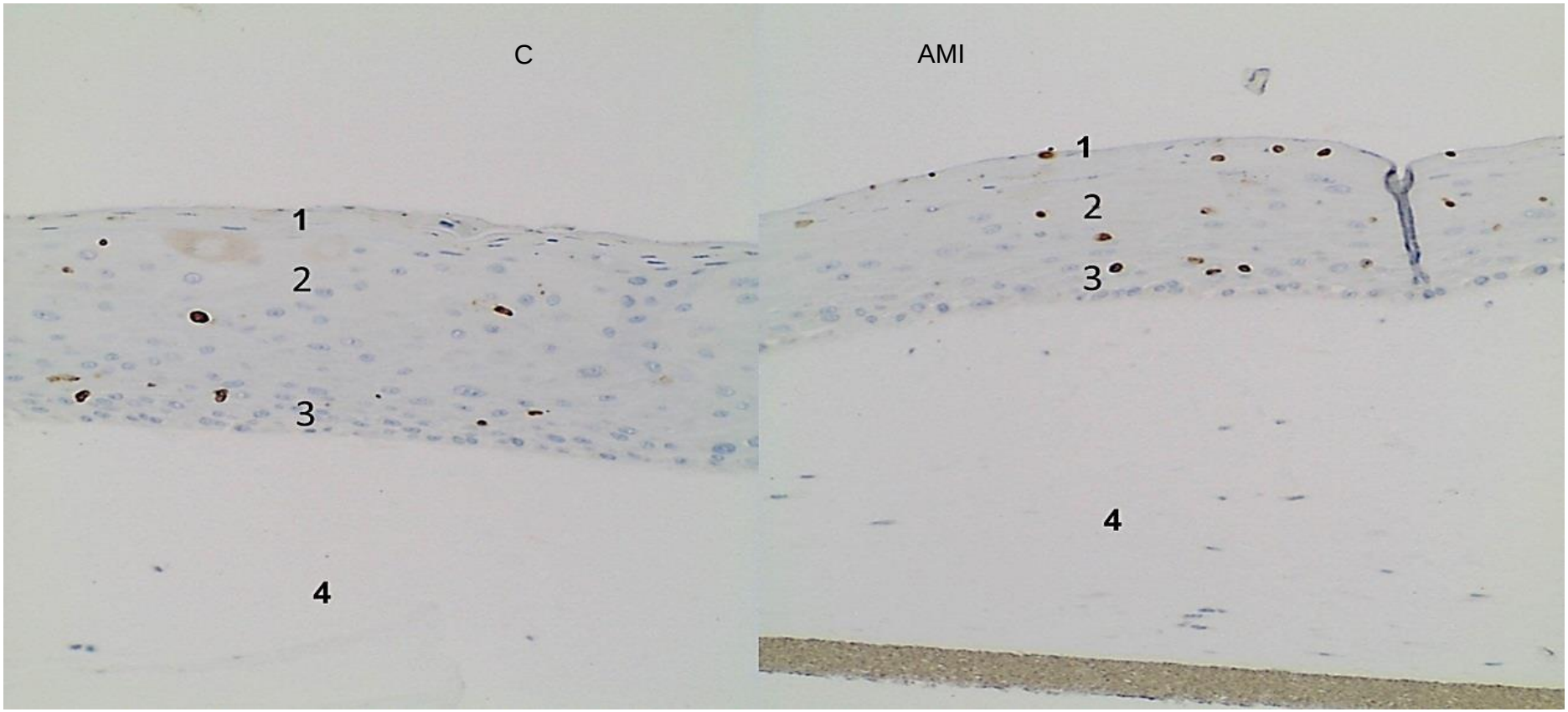


Figure 4.18: Rabbit monoclonal from R&D systems immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) for 30 min, n=3. Images shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

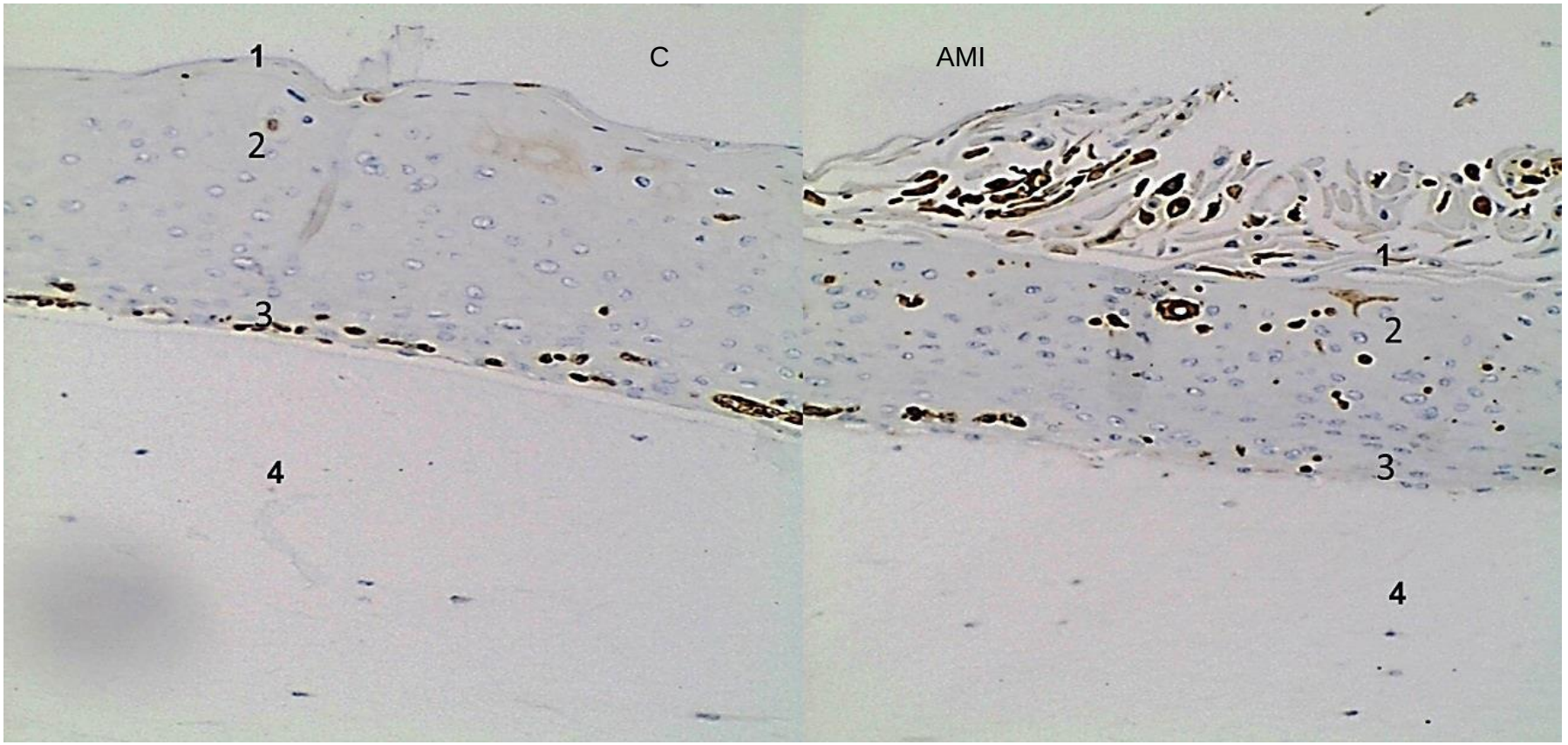


Figure 4.19: Rabbit monoclonal from R&D systems immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) for 24 h, n=3. Images are shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

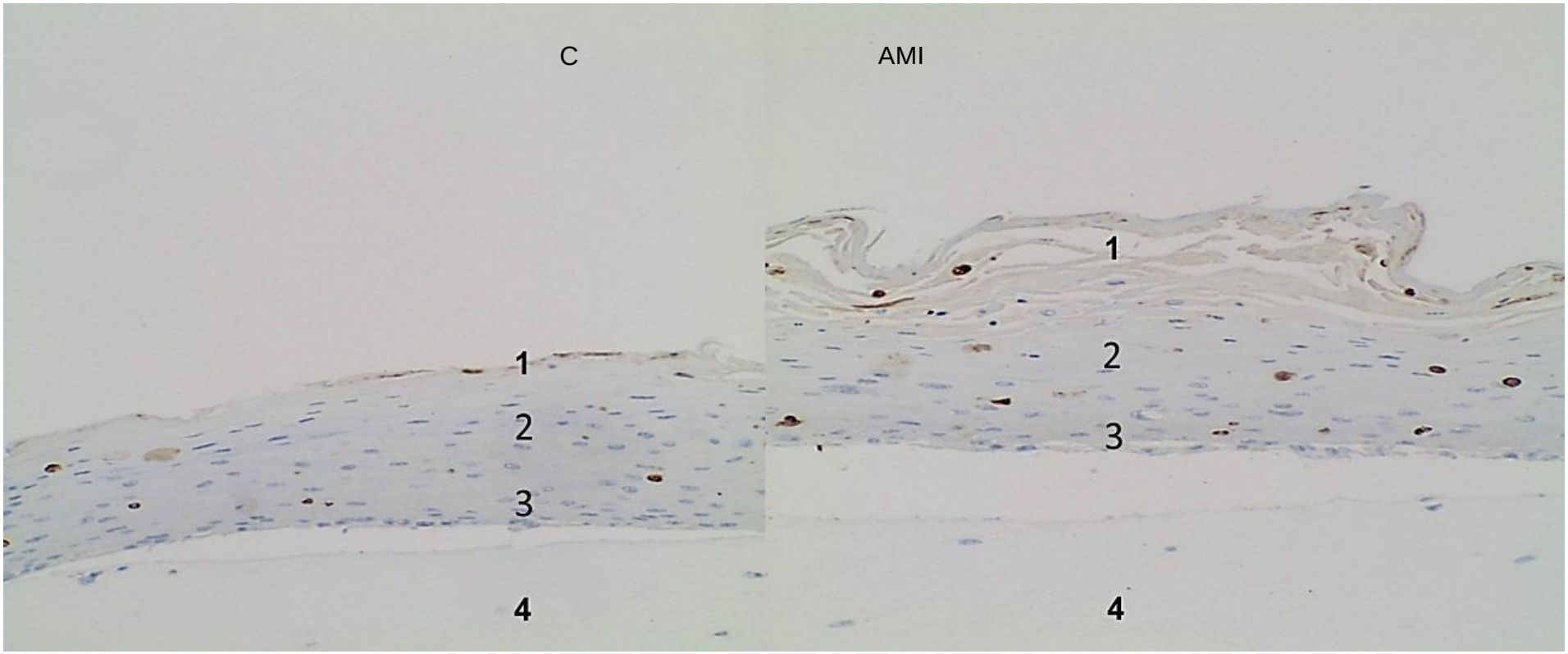


Figure 4.20: Rabbit monoclonal from R&D systems immunohistochemistry study of MatTek tissue after exposure to Amitriptyline (226 µM) twice a day (B.I.D.) for three days, n=3. Images are shown at 20 X magnification. Apoptotic cells are stained brown. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

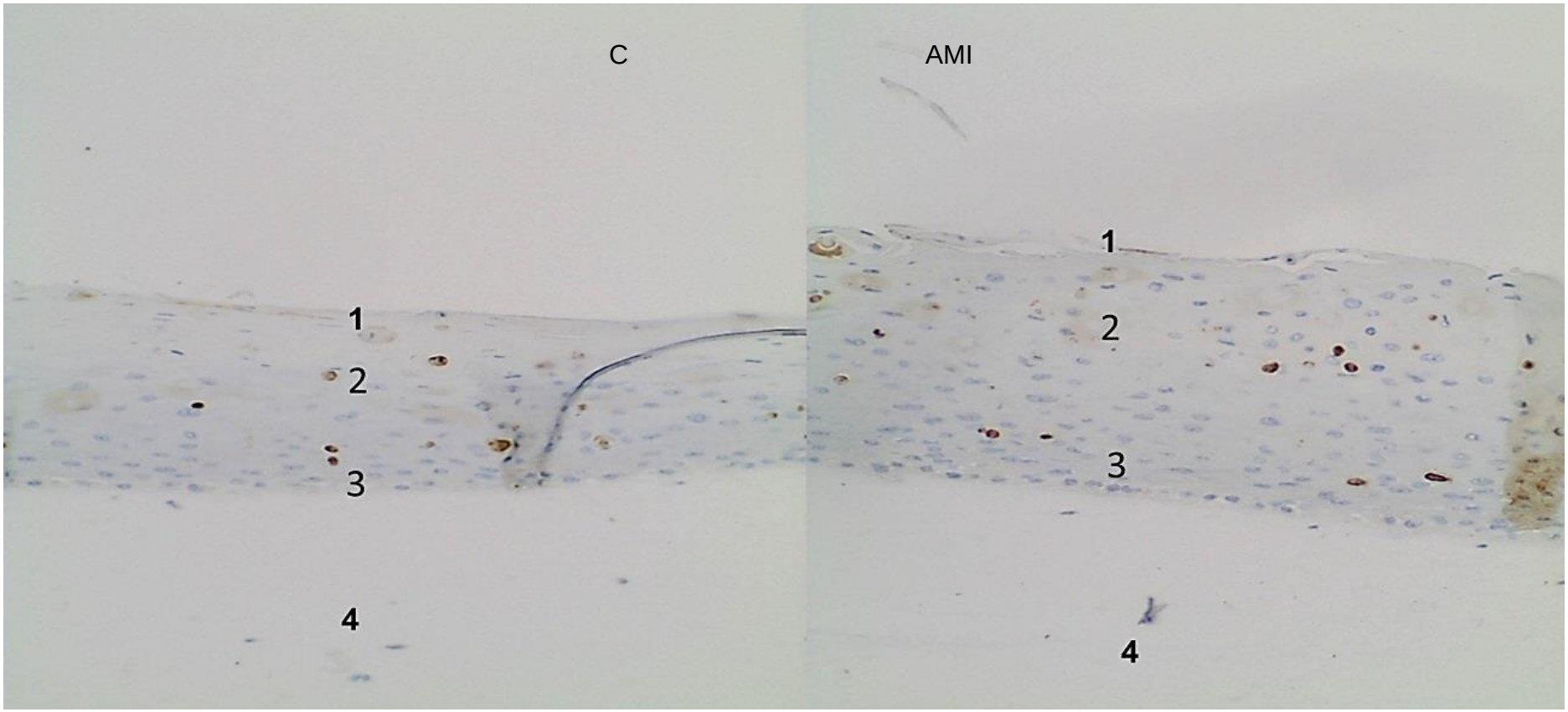


Figure 4.21: Rabbit monoclonal from R&D systems immunohistochemistry study of MatTek tissue after exposure to amitriptyline (226 µM) three times a day (T.I.D.) for three days, n=3. Images are shown at 20 X magnification. Apoptotic cells are stained brown (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

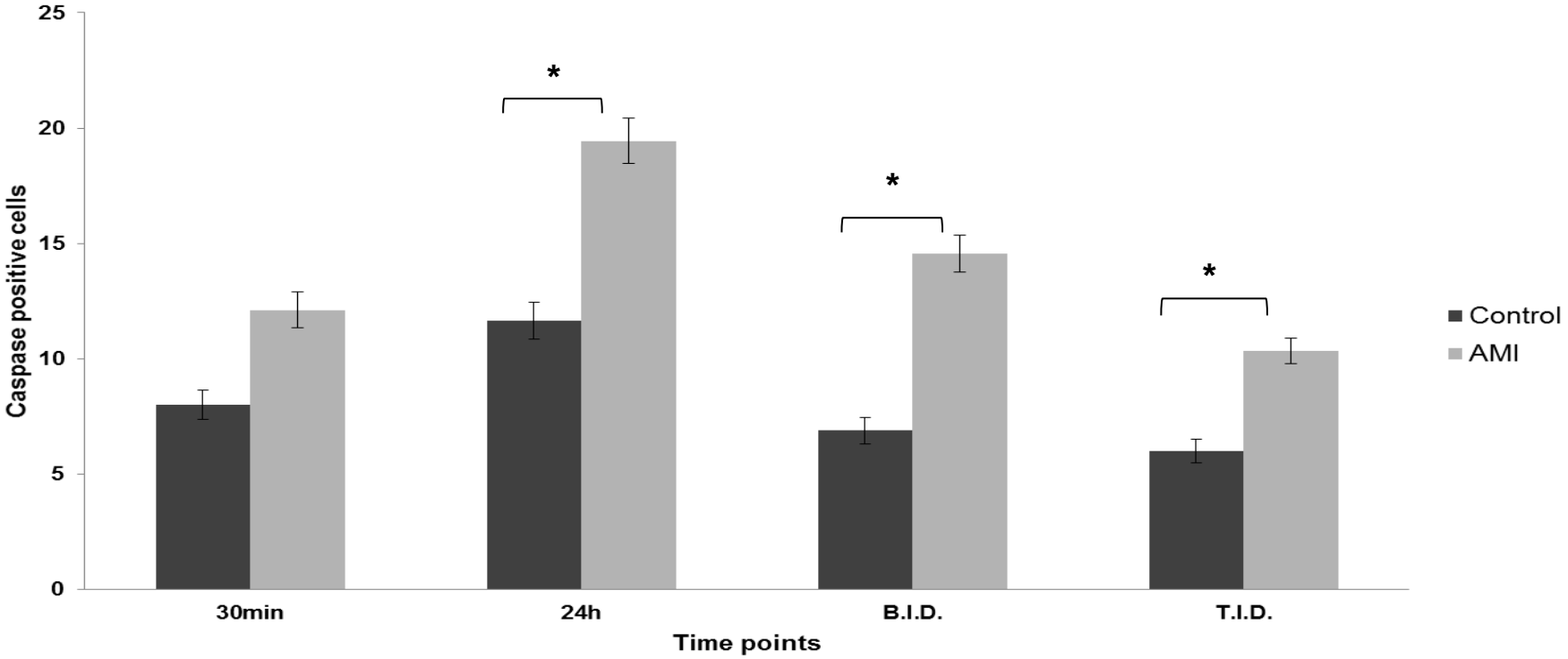


Figure 4.22: The results of the Rabbit monoclonal from R&D systems immunohistochemistry experiment of MatTek tissue exposed to amitriptyline (AMI), (226 µM) at 30 min, 24 h, B.I.D., T.I.D. Mean ± SE (n=3 tissue, n=9 images analysed and presented), the results were compared with control using parametric and nonparametric independent t-test, *p<0.05.

4.5.2 Caspase 3 level in the in-house constructed 3D model

The in-house constructed tissue models showed more apoptotic activity than MatTek model in response to amitriptyline exposure at both time points (30 min, 24 h), in general the final count was less for the second antibody compared to the first one. The count was made on 3 pictures of different parts of one tissue sample.

a: Menapath polyclonal Caspase 3 in the in-house constructed model:

In first anti-caspase 3 antibody experiments, the results of 30 min exposure of amitriptyline, showed high apoptosis count and was significant compared with control tissue ($p<0.05$) and the microscopic images showed obvious destruction in tissue layers (Figure 4.23, Figure 4.25).

When the in-house constructed tissue was exposed at the longest exposure time point (24 h) for amitriptyline, counting results were non-significant compared with control ($p<0.05$). The positive apoptotic activity a part from the main tissue strip was neglected as it may be related to the tissue preparation procedure (Figure 4.24, Figure 4.25).

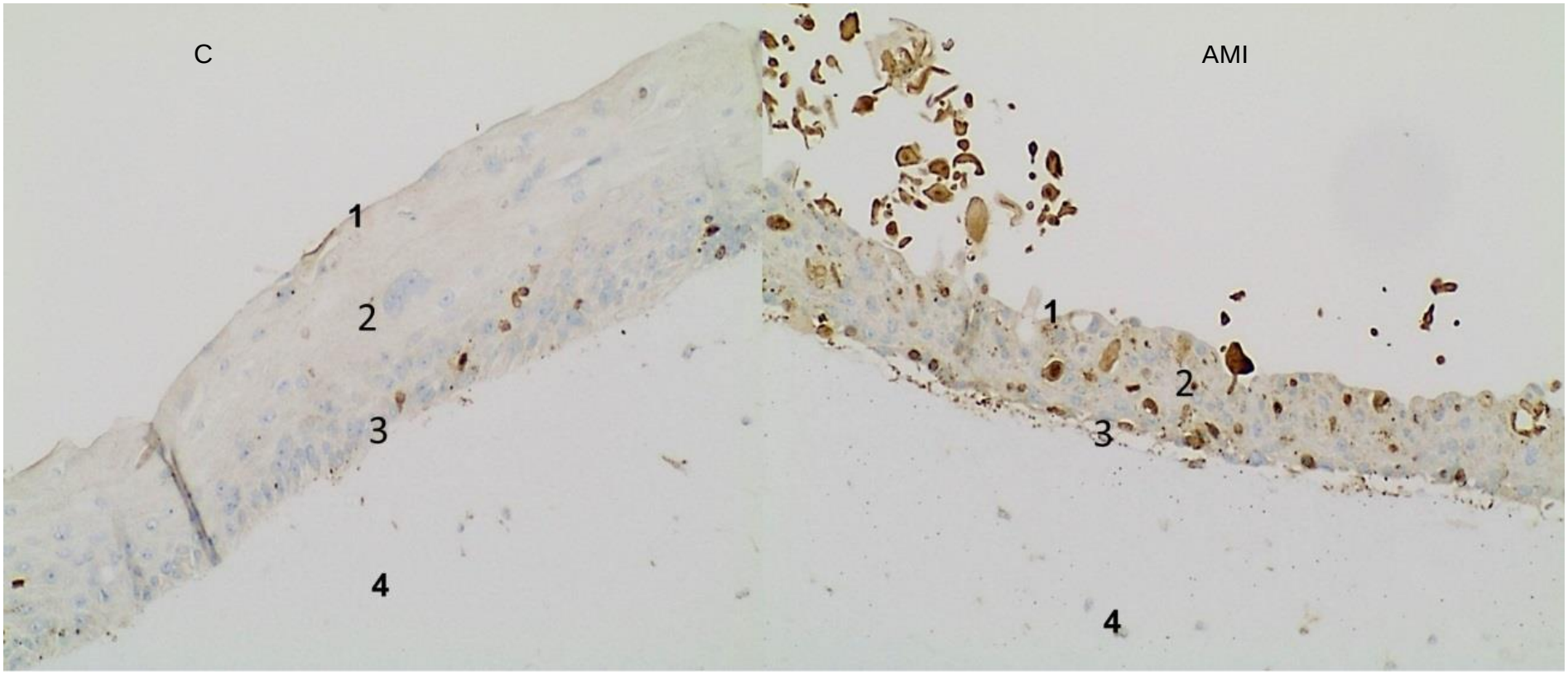


Figure 4.23: Menapath polyclonal Caspase 3 immunohistochemistry study of the in-house constructed tissue after exposure to amitriptyline (226 µM) for 30 min, n=1. Images shown at 20 X magnification.(C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

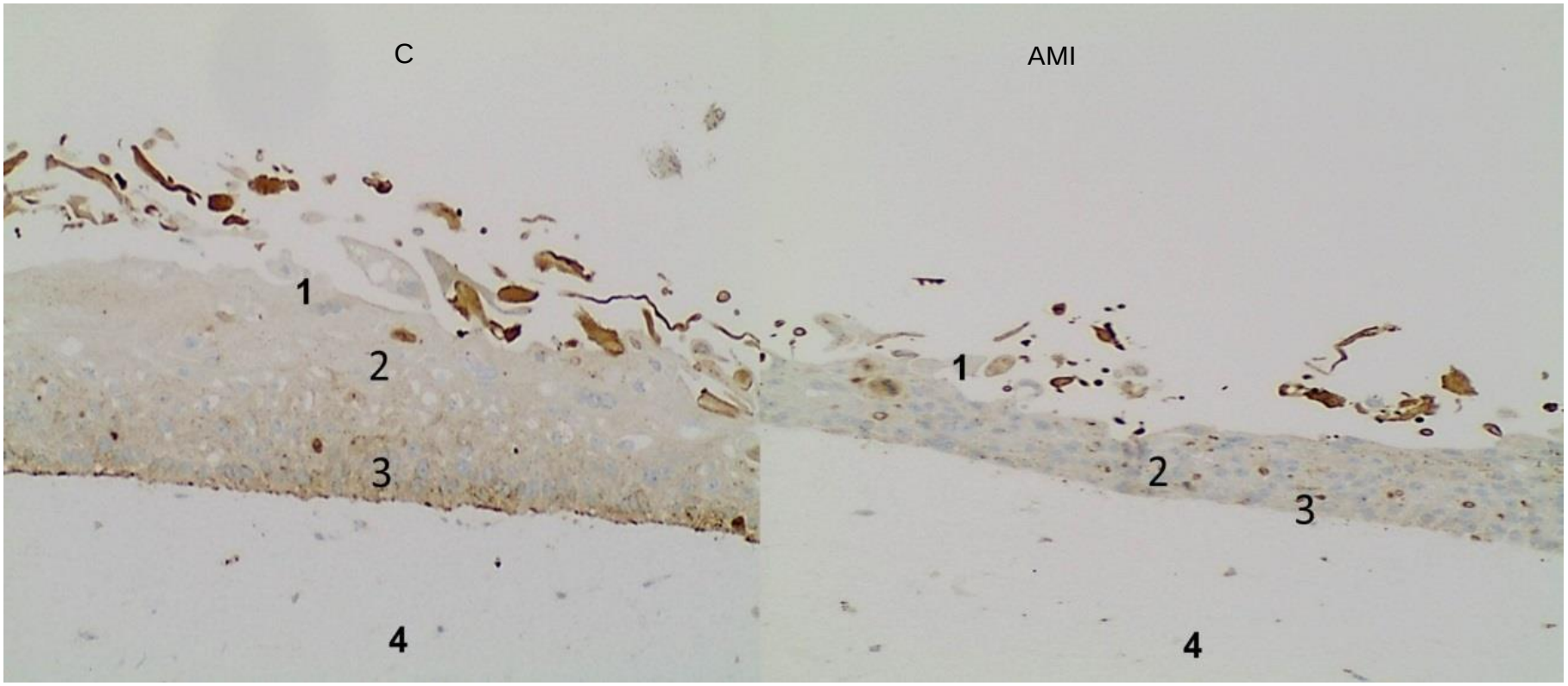


Figure 4.24: Menapath polyclonal Caspase 3 immunohistochemistry study of the in-house constructed tissue after exposure to amitriptyline (226 µM) for 24 h, n=1. Images shown at 20 X magnification. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

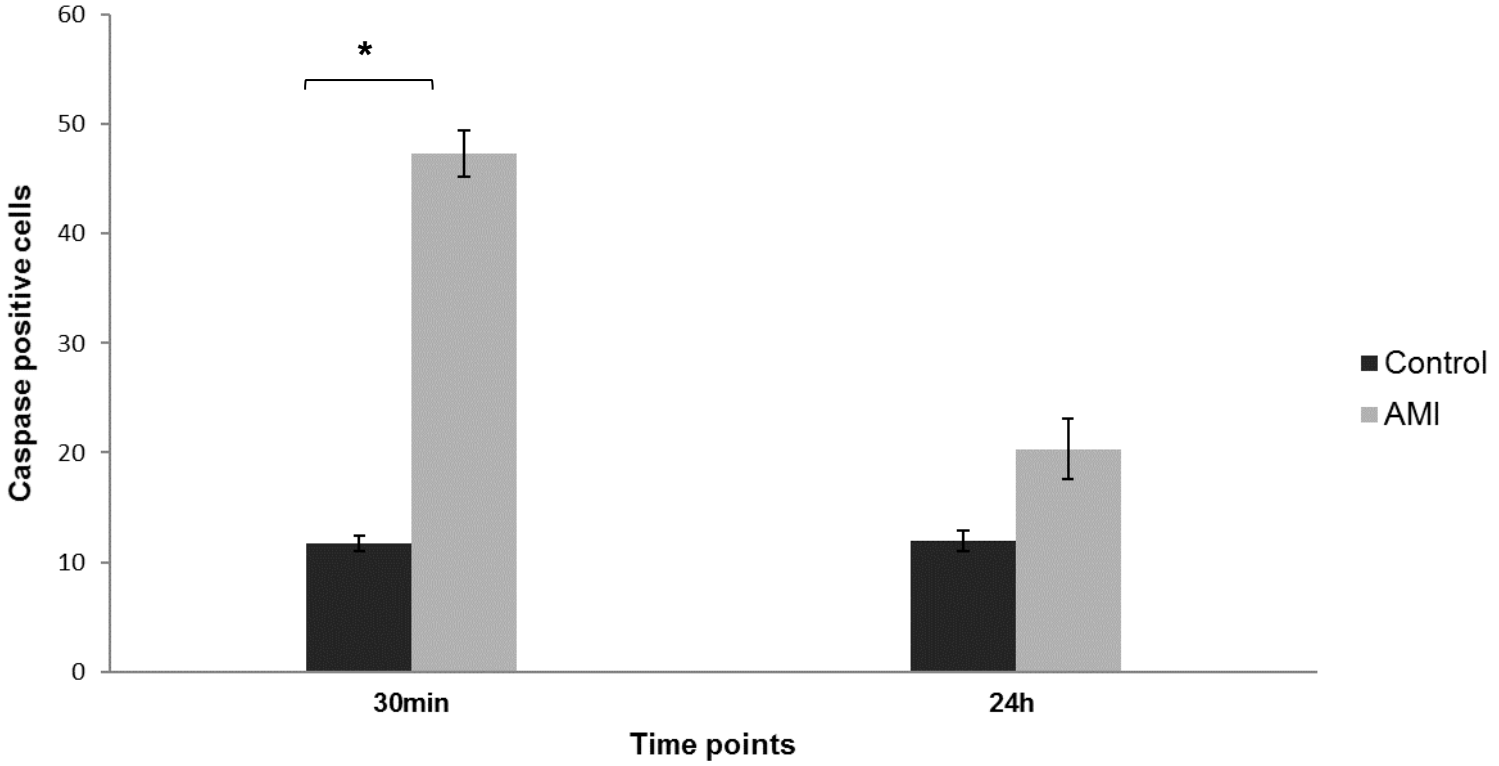


Figure 4.25: The results of Menapath polyclonal Caspase 3 antibody immunohistochemistry experiment of the in-house constructed tissue was exposed to amitriptyline (AMI) at 30 min, 24 h. Mean ± SE (n=1 tissue, n=3 images analysed and presented), the results were compared with control using nonparametric independent t-test (Mann-Whitney), *p<0.05.

b: Rabbit monoclonal from R&D systems in in-house constructed model

The same time points of exposure (30 min , 24 h) were employed for the second antibody experiments and both time points (30 min , 24 h) results were significant comparing with control tissue results ($p<0.05$); Figure 4.26, Figure 4.28 are shown 30 min time point results, Figure 4.27, Figure 4.28 are shown 24 h time point results.

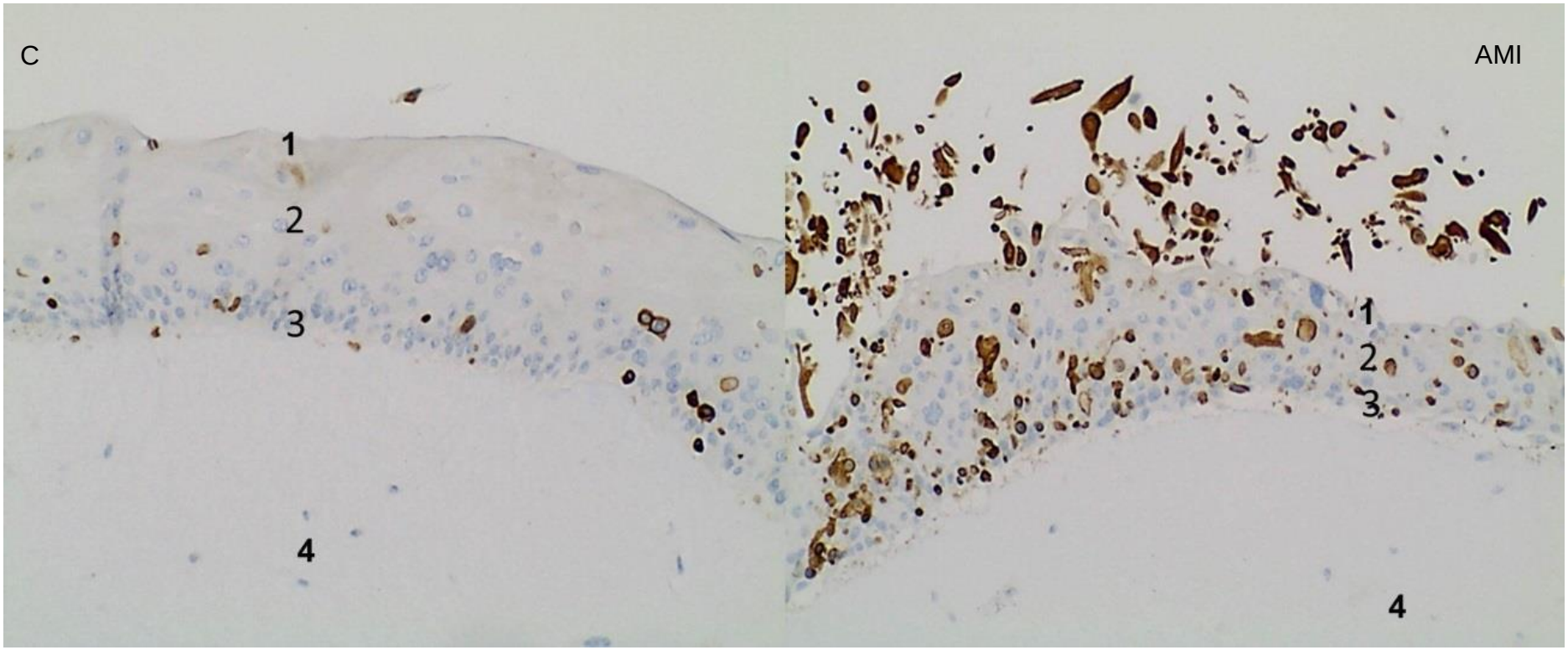


Figure 4.26: Rabbit monoclonal from R&D systems immunohistochemistry study of in-house constructed tissue after exposure to amitriptyline (226 µM) for 30 min, n=1. Images shown at 20 X magnification. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

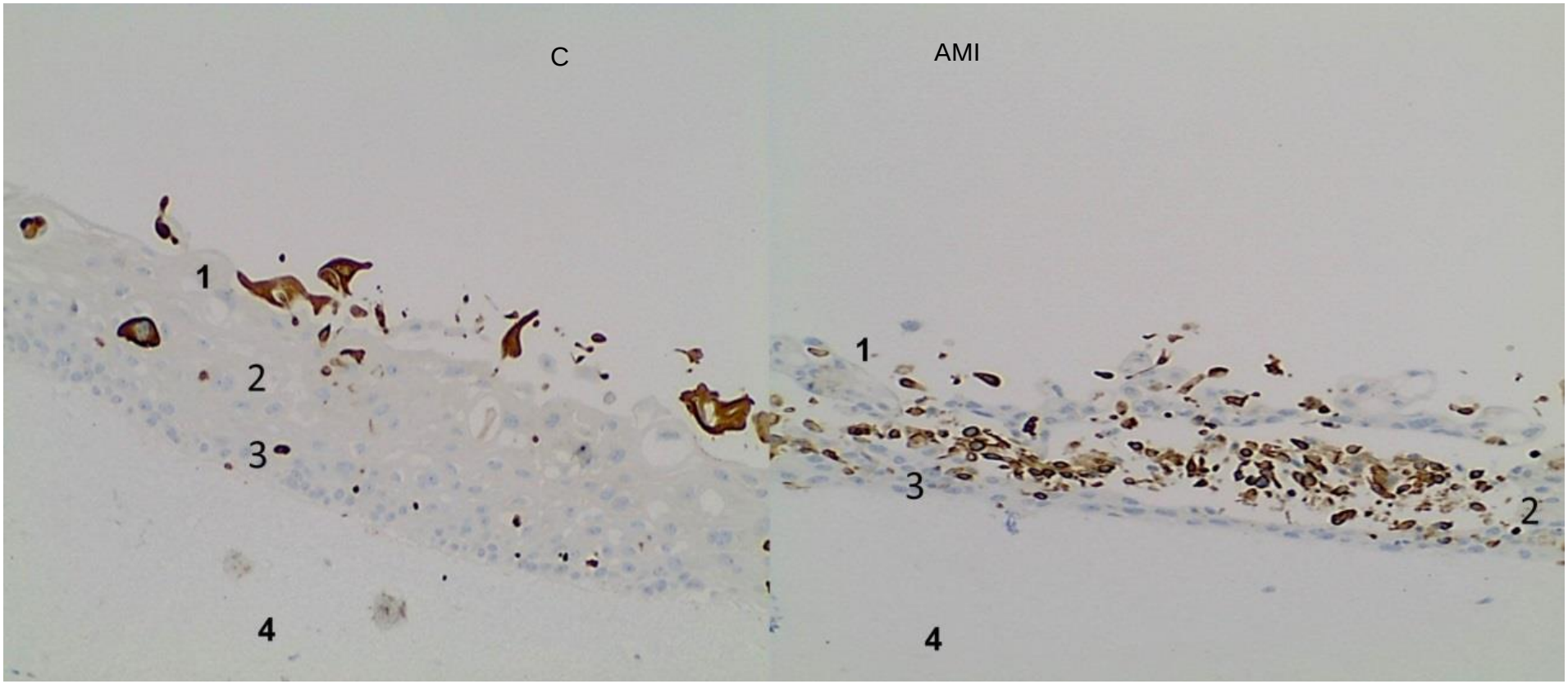


Figure 4.27: Rabbit monoclonal from R&D systems immunohistochemistry study of the in-house constructed tissue after exposure to amitriptyline (226 µM) for 24 h, n=1. Images shown at 20 X magnification. (C: control, AMI: amitriptyline, 1: keratin layer, 2: cellular layer, 3: basal layer, 4: collagen layer).

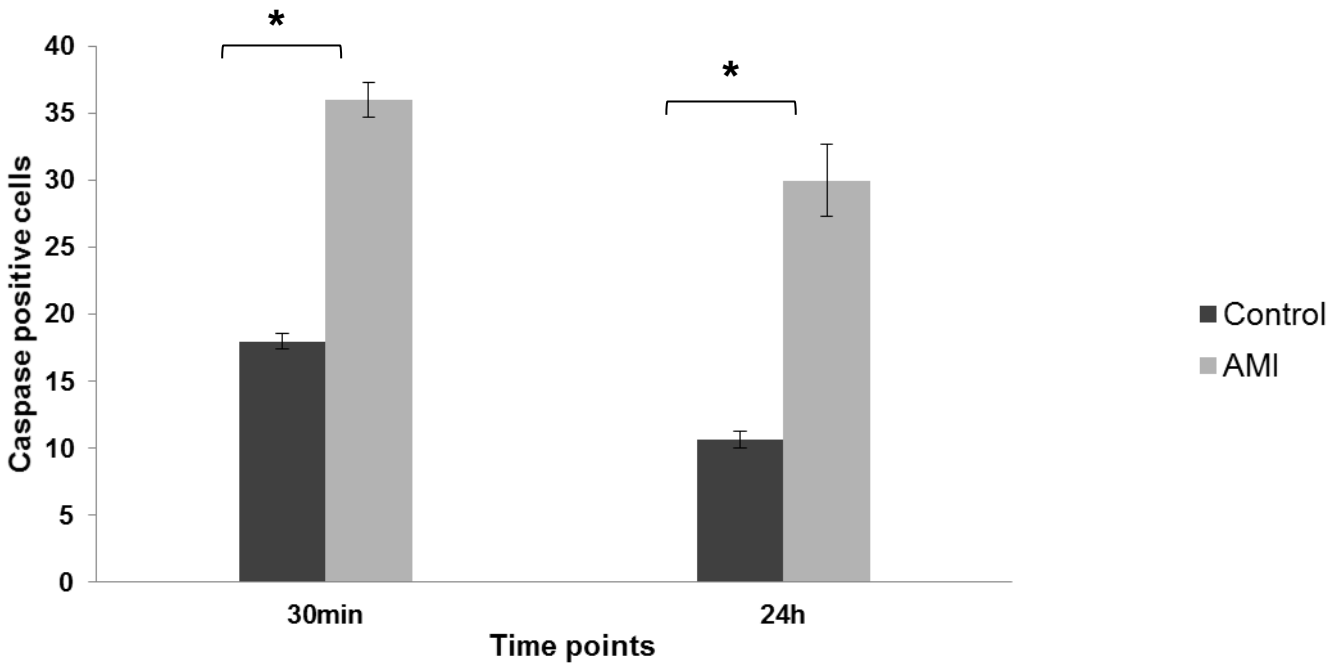


Figure 4.28: The results of Rabbit monoclonal from R&D systems antibody immunohistochemistry experiment of the in-house constructed tissue was exposed to amitriptyline (AMI), (226 µM) at 30 min, 24 h. Mean ± SE (n=1 tissue, n=3 images analysed and presented), the results were compared with control using nonparametric independent T test (Mann-Whitney), *p<0.05.

4.6 Amitriptyline penetration through the tissue

These experiments were designed to investigate whether topical treatment of amitriptyline has the ability to penetrate the mechanical barriers in the oral mucosa, as it is crucial for the drug to provoke the required effect on peripheral receptor target. Two types of experiments were conducted to examine amitriptyline drug passage through both 3D tissue models, MatTek and in-house constructed models: an immunofluorescence study using an anti-amitriptyline antibody and HPLC chemical analysis of the conditioned media collected from the lower chamber of the tissue inserts and transwell plates.

4.6.1 Immunohistochemistry study to look at amitriptyline passage through 3D tissue

Immunofluorescence studies of the presence of amitriptyline within the 3D tissue models were made using anti-amitriptyline antibody (mouse monoclonal anti-amitriptyline antibody, AbCam®, UK). Image analysis of both models revealed the presence of amitriptyline (green dots) within the tissue layers at all-time points, with the exception of 30 min exposure time in the in-house constructed model. The results of MatTek model are shown in Figure 4.29, Figure 4.30, the results of the in-house constructed model are shown in Figure 4.31.

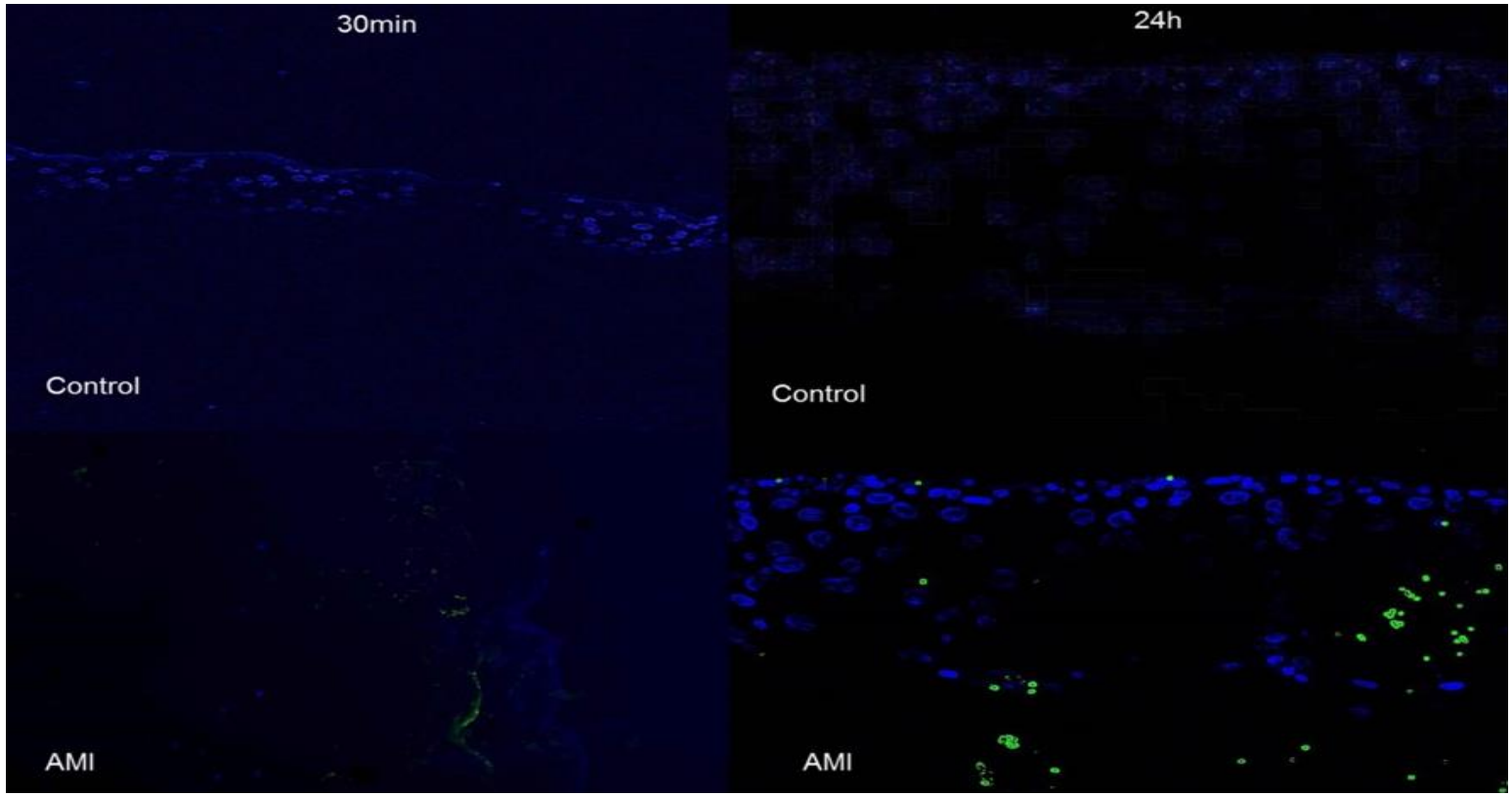


Figure 4.29: Representative images showing immunofluorescent results in the MatTek tissue exposed to amitriptyline (226 µM) for 30 min and 24h and fixed and stained with anti-amitriptyline antibody followed by secondary fluorescent antibody (anti-mouse, Alexa Flour 488) showed in green and visualized at 20 X using florescent microscope. Blue stain is DAPI (nuclei staining), Green colour is for amitriptyline, n=2.

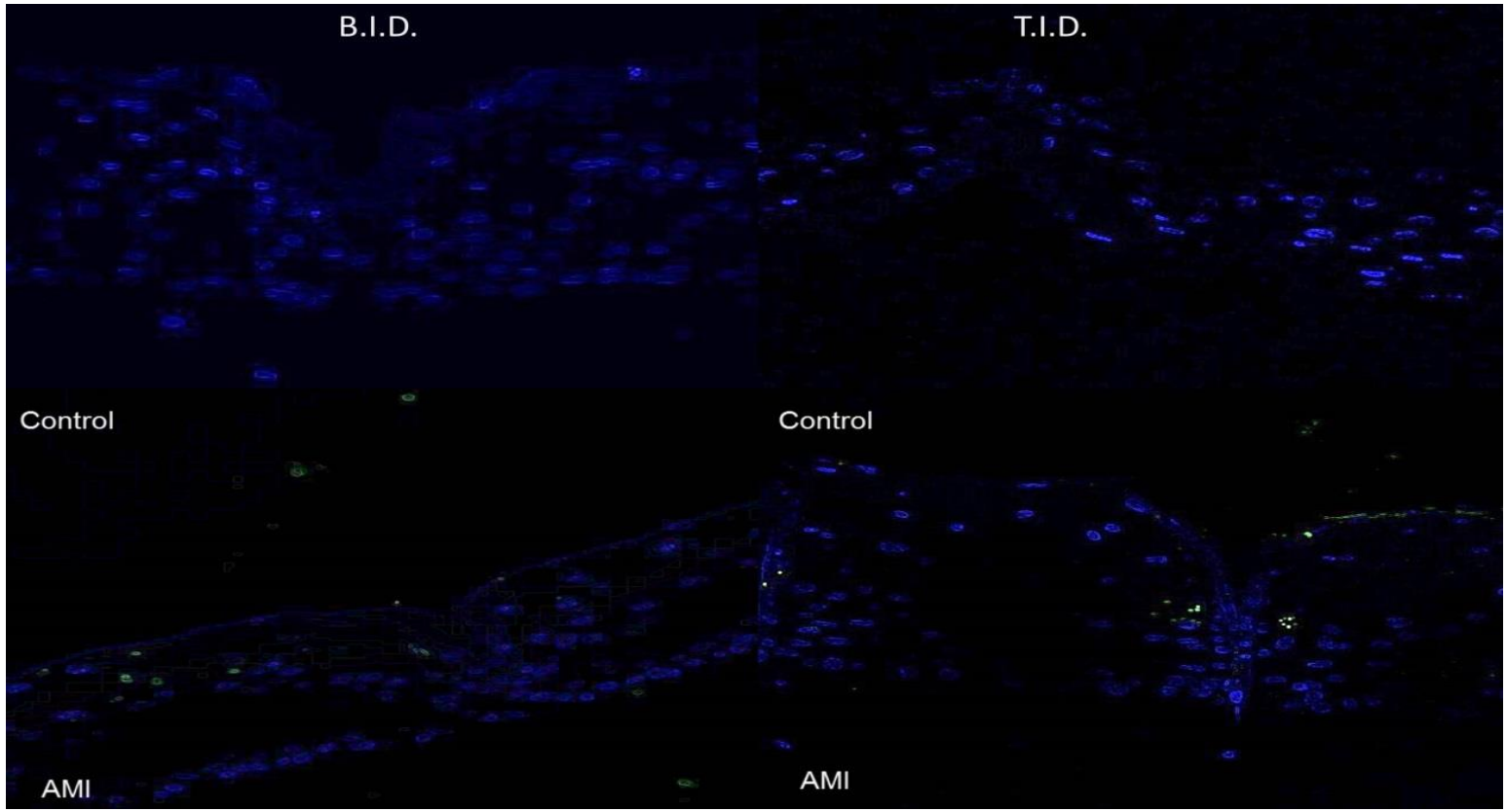


Figure 4.30: Representative images showing immunofluorescent results in the MatTek tissue exposed to amitriptyline (AMI), (226 µM) twice a day (B.I.D.) and three times a day (T.I.D.) for three days and fixed and stained with anti-amitriptyline antibody followed by secondary fluorescent antibody (anti-mouse, Alexa Flour 488) showed in green and visualized at 20 X using florescent microscope. Blue stain is DAPI (nuclei staining), Green colour is amitriptyline, n=2.

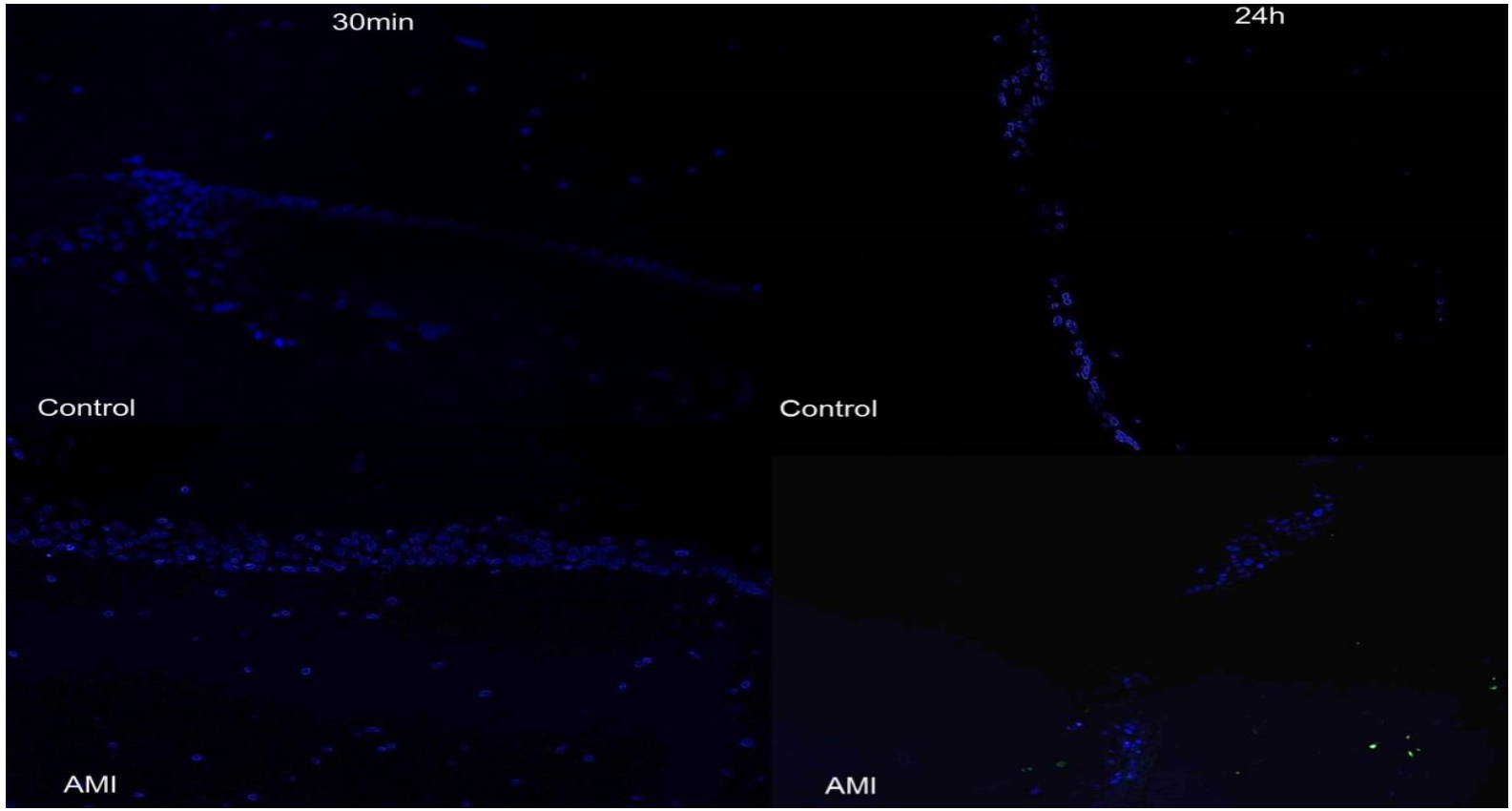


Figure 4.31: Representative image showing immunofluorescent results in the in-house constructed tissue exposed to amitriptyline (226 µM) for 30 min and 24 h and fixed and stained with anti-amitriptyline antibody followed by secondary fluorescent antibody (anti-mouse, Alexa Flour 488) showed in green and visualized at 20 X using florescent microscope. Blue stain is DAPI (nuclei staining), Green colour is amitriptyline, n=1.

4.6.2 Measurement of amitriptyline infiltration through tissue by Chemical analysis (HPLC)

The conditioned media were collected from the lower chamber of the 3D model after all exposure times and analysed by HPLC to find how much drug is able to pass through the tissue.

The MatTek model tissue barrier was more developed. After short exposure time of 30 min, the levels of amitriptyline measured in the media were similar to control, where undetected concentration of the tested drug was the results of the HPLC. The results of the remainder time points are shown in Table 4.2 and Figure 4.32.

Higher detected concentrations of amitriptyline were reported after 24 h exposure time in the MatTek model, which is reasonable with such long exposure time. The repeated exposure (B.I.D. and T.I.D.) results are actually 30 min exposure results after 3 days of continual exposure, and these were lower than 24 h but considerably higher than single 30 min exposure.

The chemical analysis of the in-house constructed tissue model, showed how the differences in tissue construction affects tissue barrier integrity and that had an obvious effect on the drug passage, when the final concentrations were nearly the same in all time points results except the control. Table 4.3 and Figure 4.33, show HPLC results of the in-house 3D model.

Time points	Amitriptyline concentration measured by HPLC µM	Relative to applied concentration %
30 min	0.085	0.26
24 h	6.65	20.6
B.I.D.	0.6	1.86
T.I.D.	0.89	2.75

Table 4.2: The results of HPLC (chemical analysis) of the MatTek tissue exposed to amitriptyline concentration (226 µm) for 30 min, 24 h, B.I.D. and T.I.D. time points, (n=3).

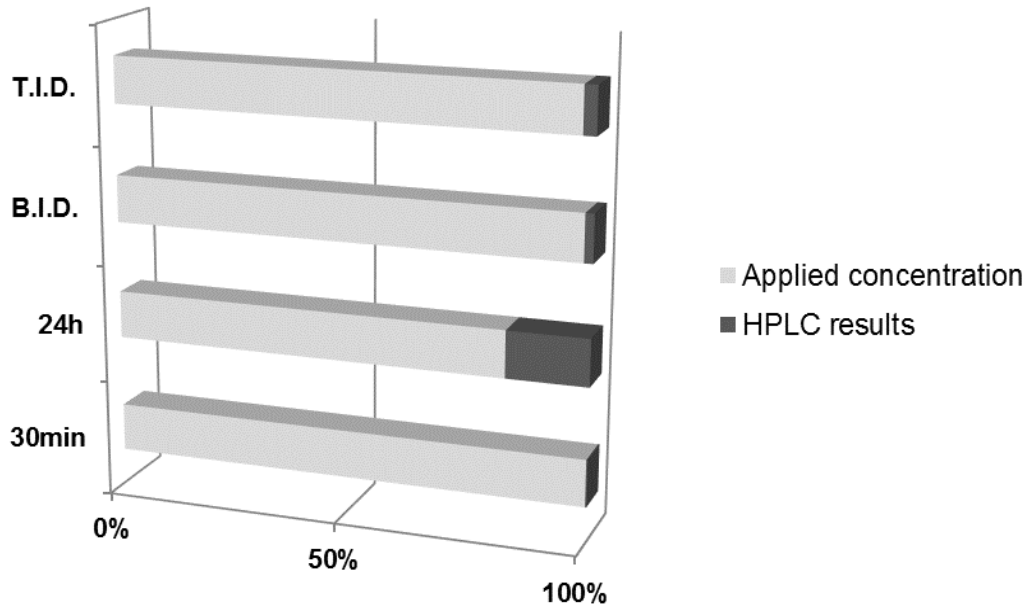


Figure 4.32: HPLC results of MatTek tissue exposed to amitriptyline for 30 min, 24 h, B.I.D. and T.I.D. time points, (n=3).

Time points	Amitriptyline concentration measured by HPLC µM	Relative to applied concentration %
30 min	40.46	89.5
24 h	40.14	88.8

Table 4.3: The results of HPLC (chemical analysis) of the in-house constructed tissue exposed to amitriptyline concentration (226 µm) for 30 min and 24 h time points, (n=2).

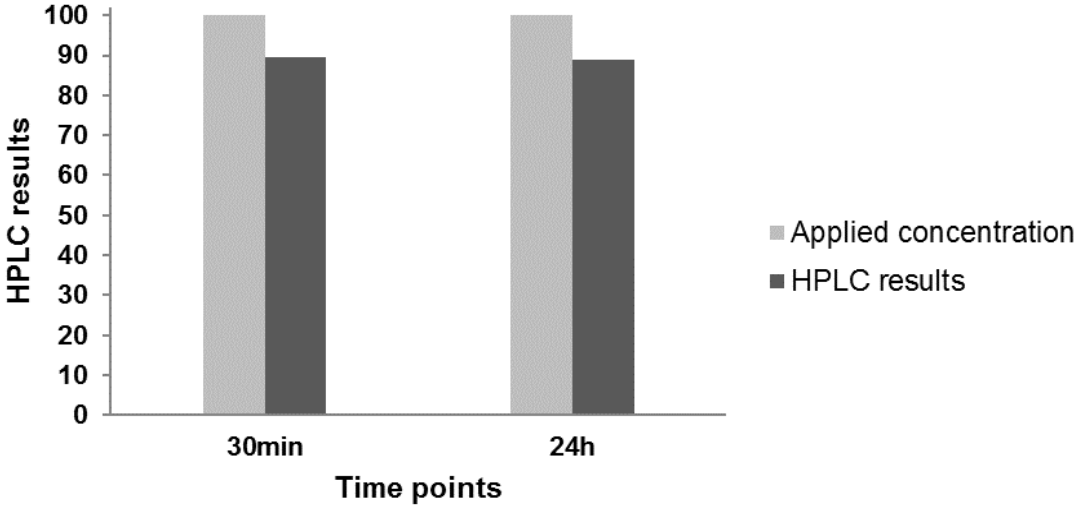


Figure 4.33: HPLC findings of the in-house constructed model exposed to amitriptyline for 30 min and 24 h time points , n=2.

4.7 Discussion

As a preclinical testing pathway for the drug development process, monolayer cell culture (2D) and its cellular assays are relatively inexpensive, fast and an accessible representation of the *in vivo* environment (Zou *et al.*, 2007). Recently, however, concern has been expressed regarding the limitations of 2D cell culture as a model for the *in vivo* tissue environment. The introduction of a 3D tissue model has, therefore, been suggested to be an appropriate, more sophisticated physiological substitute for both human *in vivo* environment and animal studies. This type of model gives advantage such as the possibility to study pathophysiology, treatment safety and prognosis with better *in vivo* expectations and less chance of misleading data as in 2D studies (Griffith and Naughton, 2002; Maltman and Przyborski, 2010). The introduction of the 3D tissue model to study the biocompatibility of many dental materials have found to be promising with an *in vitro* response found close to *in vivo* biological responses using 3D cell culture (Klausner *et al.*, 2007; Moharamzadeh *et al.*, 2012).

Unlike monolayer cell studies, 3D *in vitro* studies are more representative and realistic as a model for the *in vivo* environment, particularly regarding the inter-cellular and cellular-matrix orientation and interaction (Maltman and Przyborski, 2010). Different viability measures showed that the response of 3D cell culture is less negative when compared to 2D cell culture results. Viability reduction was about 50 % different and inflammatory cytokines levels were under expressed when same stimuli applied on 3D tissue models (Sun *et al.*, 2006; Moharamzadeh *et al.*, 2012).

The viability assay used in this chapter is alamarBlue®, it demonstrates the cellular viability through chemical reduction of Resazurin. Using alamarBlue® has many advantages especially for the expensive, time consuming, type of cell culture as it did not affect the tissue or cellular integrity and this helps the researcher in being able to conduct further research and investigation on the same cell culture. This fact informed our decision to use alamarBlue® rather than a MTT assay, as we would lose the tissue for further downstream applications and just produce the viability measurement without having the

possibility of using the tissue for further designed investigations such as H&E staining, immunohistochemistry or immunofluorescence studies (Colley et al. 2013).

In the monolayer cell culture viability study, there is a pre-estimation experiment for exposure time and cell count to be employed for the viability study (Serotec®, 2015). However, that was difficult to apply in 3D tissue as using a group of tissues for estimation is rather expensive and time consuming, in addition, the seeding cell count at the time of 3D tissue construction is the same but it is not necessary the same eventually, after the model completion. Accordingly, after amitriptyline exposure, 3D tissue was submitted for alamarBlue® test for 2 h and 6 h in in-house constructed and MatTek models respectively according to observation and previous studies (Colley et al. 2013).

The amitriptyline concentration used was as determined by the research presented in Section 3.4. Time points implemented were 30 min and 24 h in both models (MatTek and in-house constructed) and repeated exposure [twice a day (B.I.D.) and three times a day (T.I.D.) for three days] in MatTek models only. The application of repeated exposure was designed to reflect the effect of repeated toxicity and to explore if there is a reversal toxicity as recommended in toxicity guidelines (EMA, 2010). The experiment time (3 days) was shorter than classic clinical course for neuropathic pain topical treatment (Lynch *et al.*, 2005b), but is limited to the expected 3D model shelf life according to manufacturer's instructions (MatTek, 2015). The incorporation of repeated exposure time points was a key factor to build a better understanding of amitriptyline's apoptotic effect, as high apoptotic activity was found compared with single time exposure.

In the MatTek model, the tissue barrier integrity was investigated and approved using trans-epithelial electrical resistance (Klausner *et al.*, 2007; MatTek, 2015). From the amitriptyline exposure observations, culture media did not pass readily through the tissue surface, so alamarBlue® solution needed more time to penetrate through the tissue superficial keratin layer and cellular layer to measure cellular viability. After amitriptyline exposure, the MatTek tissue

models were exposed to alamarBlue® for 6 h before plate reading and this was still within an optimum alamarBlue® protocol (4 h-8 h), (Serotec®, 2015).

For the in-house constructed model, the alamarBlue® was exposed for 2 h as this was long enough to penetrate the whole tissue thickness (Colley *et al.*, 2013). The chemical barrier is less developed in the in-house constructed tissue and there was some shrinkage in the supporting collagen that affected the mechanical barrier integrity of the constructed tissue and eventually simplify media and amitriptyline passage through the tissue from tissue apical surface to lower chamber.

Viability results for both tissues models showed a reduction in viability after repeated time points and 24 h time points in MatTek and in-house constructed models respectively, but these reductions were still non-significant compared with control. The results of 3D model constructed in Newcastle oral biology laboratory were non-significant for 30 min and 24h exposure time points similar to the results of MatTek model (Figure D.1). However, there was a significant difference in the B.I.D. time point in MatTek model but it this was a small reduction in viability compared to the control. This could be related to the type of assay which may be reduced by any part of tissue covering up the difference in viability especially if we are talking about short exposure; however, we still can find as in Figure 4.1, that reduction in viability occurred after repeated exposures (B.I.D. and T.I.D.) indicating that this may occur in the *in vivo* situation.

Amitriptyline cytotoxicity was measured using LDH assay, which measures lactate dehydrogenase enzyme as representative of cell lysis. Cytotoxicity results in the conditioned media showed only a significant difference between treatment and control tissues at 24 h exposure time for the MatTek models. The results of in-house constructed model, showed a significant difference when compared to control at both time points (30 min and 24 h). The results of model constructed in Newcastle revealed significant difference at 30 min time point only (Figure D.2). LDH experiments did not reveal high toxicity may be due to the repeated washing after each exposure in the case of repeated time points of exposure might affect the expression of lactate dehydrogenase eventually in the

conditioned media. It does, however, appear that amitriptyline has a cytotoxic effect in both models.

The variations in the readings between time points and tissues and between different colorimetric assays, dictate the need for further research with more robust findings in order to confirm or deny the risk to oral mucosa that amitriptyline represents when applied topically. The results also raise a question about the suitability of colorimetric assay for 3D tissue culture. Both methods do, however, indicate an effect of amitriptyline on 3D tissue culture and that it is potentially a negative effect.

Amitriptyline's effect on 3D tissue cellular construction was further investigated using H&E slides. Except for slight findings in the MatTek model at T.I.D. time point, H&E immunohistochemistry did not show any gross morphological changes, or severe damage through the treatment groups compared to the control images for both tissue types and all exposure time points.

Amitriptyline has been found to cause disruption in mitochondrial membrane potential following increase of production of reactive oxygen species. The resulted activation of caspase 3 pathway is through release of cytochrome c that leads to mitochondrial mediated apoptosis (Green and Reed, 1998; Lirk *et al.*, 2006a; Bautista-Ferrufino *et al.*, 2011). Two types of anti-caspase 3 antibody were used to investigate apoptotic activity resulting from amitriptyline exposure. The results showed an obvious apoptotic effect in the slide images. The amount of apoptotic activity in treated group in long exposure (24 h) and long-term exposure (B.I.D. and T.I.D.) was significantly higher compared to the control. These results demonstrate that the number of dead cells through apoptosis is increased with repeated exposure to the target drug. This is important as this process mirrors the clinical application procedure of the drug for chronic pain, requiring repeated exposure topical treatment. The results were significant in both tissue types in most exposure time and highest in the repeated exposure (B.I.D., T.I.D.) and long-time exposure (24 h).

The MatTek model results were more consistent and potentially reliable through the investigations in this chapter. This may be related to the type of cells used

which were primary cells from healthy volunteers made using first or second passage. The cell line models used to construct the in-house model were from dysplastic samples where the cells behave in a more dysplastic way losing some of the normal gingival development and characteristics. In addition, the type of gingival cells (fibroblast cells) representing the supporting submucosa fibrous tissue, was less viable, causing slow reproducing and further shrinkage of the supported collagen.

Previous studies that investigated post-amitriptyline exposure related nerve damage, were thoroughly approved using animal studies. In these studies, amitriptyline was applied directly onto the nerve trunk and using concentrations similar to those applied clinically in NP treatment course (Estebe and Myers, 2004; Barnet *et al.*, 2005; Lirk *et al.*, 2006b). While many clinical trials have used higher concentrations, they did not report any epithelial or nerve damage. This raises the question about how amitriptyline is able to pass through the tissue, particularly with short exposure time points and how much of the drug is expected to reach the target nerve endings. Estebe and Myers (2004) reported that the detergent effect of amitriptyline caused irreversible nerve degeneration at concentrations higher than 0.3 %, (10 mM). However, clinical studies which applied amitriptyline topically with high concentration [1.5-10 %, (50-300 mM)] on skin and mucosa reported no signs of nerve degeneration; the only reported effect was a temporary inflammatory response (Padilla *et al.*, 2000; Lynch *et al.*, 2005a; Dualé *et al.*, 2008; Kopsky and Keppel Hesselink, 2012).

Amitriptyline passage through the tissue was investigated in this work using chemical analysis (HPLC) and immunofluorescence investigation. The results showed the presence of the amitriptyline within the tissue at most of the time points using the anti-amitriptyline antibody, while results of chemical analysis of lower chamber amitriptyline concentrations were varied between MatTek and in-house model.

The in-house constructed tissue suffered a collagen shrinkage that affected the barrier characteristics of the tissue which is shown in the results, where 30 min and 24 h exposure results were the same. For the MatTek model, the HPLC results varied according to exposure time, negligible concentrations of

Amitriptyline were calculated after 30 min exposure while the 24 h exposure showed a higher rate of drug passage through the tissue, with the repeated exposure (B.I.D. and T.I.D.) results showing that the repeated exposure for 30 min affected the tissue integrity and there may even be cellular death disrupting the barrier characteristics and integrity. The results show that there were no measurable changes in tissue and cellular morphology, but that amitriptyline still has the ability to penetrate through epithelial surface. More investigations are needed to disclose if the increase in penetration ability of amitriptyline is due to cellular death, change in the intercellular junction characteristics or both of them.

The lipophilic affinity and para-cellular penetration characteristic of its tertiary amine structure (Sudoh *et al.*, 2003) give the amitriptyline an adjuvant penetrative capacity. However this is not completely accurate, as amitriptyline passage after 30 min was negligible, but with more tissue integrity disruption as happened after repeated application, a higher concentration was found in the lower chamber.

Dahlin *et al.* (1999) found that amitriptyline exposure affects cytoskeletal orientation and increases para-cellular permeability even at low amitriptyline concentrations, resulting in disruption of tight intercellular junction structure and function. In addition, we found (in Chapter 3) that amitriptyline exposure affects cellular morphology and attachment, and the collective cytoskeleton orientation of the monolayer cell culture. Further, in this chapter, repeated exposure of low concentrations (226 µM) at short time point (30 min) affected the intercellular tight junctions and increased tissue permeability, which may explain the increase in tissue permeability to amitriptyline at repeated exposure time points of exposure as found by HPLC.

Chapter: 5 The expression of peripheral receptors in human gingival tissue

5.1 Introduction

The complexity and the role of peripheral receptors in the pathophysiology of neuropathic pain has become of more importance given the recent expansion in research concerning topical management strategies in NOP (Padilla *et al.*, 2000; Merrill, 2004; Cairns, 2009b). Animal, *in vitro* and human functional studies (Doak and Sawynok, 1995; Sawynok and Liu, 2003; Hirai and Hama, 2014), have been designed to decode the peripheral process of pain and to find possible changes in receptor expression and excitability to understand their role in pain pathophysiology. The studies examining receptor expression and excitability in chronic pain have revealed the function of a group of receptors and/or ion channels that are involved in orchestrating peripheral persistent pain and also other sensation changes such as hyperalgesia and allodynia. These sensory changes have been identified to associate with over and under excitation or expression of specific receptors, during different types of neuropathic pain (Sawynok, 2003; Sawynok *et al.*, 2005; McGivern, 2009; Sawynok, 2009; Sessle, 2009). Topical administration of NMs in NOP, offers the opportunity to target these receptors, achieving a fast response and avoiding systemic route complications (Heir *et al.*, 2008; McDougall, 2011; Haribabu *et al.*, 2013; Casale and Mattia, 2014).

For this preliminary study and as representative of trigeminal innervation, a single anonymous sample of human gingival tissue was analysed for genetic investigations of peripheral receptors. Ethical approval (Newcastle & North Tyneside 2 Ref 7/Q1003/41) and written consent was obtained before a gingival tissue biopsy was excised from the palatal area as part of a routine treatment plan for canine exposure for orthodontic treatment purposes. The tissue was kept directly in the RNA*later*® (Ambion®, Paisley, UK), solution before further storage at -80 °C until the RNA extraction procedure (Section 2.13.2). The gingival tissue sample was used to investigate the expression at the mRNA level, of a group of receptors and ion channels identified as the most expected targets for the three investigated NMs in his study: amitriptyline, carbamazepine

and gabapentin. The expression of these receptors was also investigated in parallel mRNA extracted from a neuroblastoma cell line (SH-SY5Y). The results were further investigated in RNA extracted from an oral keratinocyte cell line model (OKF6-TERT1), to investigate whether there is cross expression between trigeminal nerve endings in the gingival biopsy and oral keratinocyte cells.

The receptors of interest were identified after thorough literature search (Section 1.6). Oligonucleotide primers were designed using NCBI Blast package (Section 2.14.4) or ordered directly from commercially available oligonucleotide options (Life Technologies Ltd, UK).

Standard reverse transcriptase (RT) procedure (Section 2.14) was used to prepare the complementary DNA (cDNA) samples from the gingival and SH-SY5Y RNA samples. Expression of the target receptors' genes was investigated using polymerase chain reaction (PCR) and specified primers. The results were visualised by gel electrophoresis (2.14.3) and all positive results were confirmed by sequencing services (Eurofins Genomics, UK). The selected targets for genetic investigations are shown in Table 5.1.

A housekeeping gene (GAPDH) primer was also used to investigate the integrity of the cDNA samples and to ensure quality. The band expression was strong for the all samples indicating the cDNA was adequate.

Receptor type	Gene	Phenotype	Drug in action	References supporting receptors' inclusion
Adenosine receptor	ADORA2A	Adenosine A2 receptor	Amitriptyline Carbamazepine	(Sawynok *et al.*, 2005) (Lee and Abrahams, 2008)
Glutamate receptors (NMDA channel)	GRIN1	Glutamate ionotropic receptor NMDA type subunit 1	Amitriptyline Carbamazepine	(Cairns, 2009) (Hough *et al.*, 1996)
	GRIN2A	Glutamate ionotropic receptor NMDA type subunit 2A		
	GRIN2B	Glutamate ionotropic receptor NMDA type subunit 2B		
	GRIN2C	Glutamate ionotropic receptor NMDA type subunit 2C		
	GRIN2D	Glutamate ionotropic receptor NMDA type subunit 2D		
Sodium channels	SCN3A	Sodium channel, voltage-gated, type III, alpha subunit (Na_v 1.3)	Amitriptyline Carbamazepine	(Esser and Sawynok, 1999) (Lee and Abrahams, 2008)
	SCN9A	Sodium Channel, Voltage-Gated, Type IX, Alpha Subunit (Na_v. 1.7)		
	SCN11A	Sodium Channel, Voltage-Gated, Type XI, Alpha Subunit (Na_v 1.9)		
Calcium channels	CACNA2D1	Calcium voltage-gated channel auxiliary subunit alpha2delta 1	Gabapentin	(Maneuf *et al.*, 2006)

Table 5.1: List of peripheral analgesic receptors for amitriptyline, carbamazepine and gabapentin were investigated in the gingival tissue and SH-SY5Y cell line samples at mRNA level using PCR standard technique.

Name and accession no.	Primer sequence	Length (bp)	Annealing temp (°C)
ADORA2A NM_001278497.1	5'-$_{809}$TCCCCTTTGCCATCACCATC$_{828}$-'3 5'-$_{1414}$AGTCGGGGCAGAAGAAAGTG$_{1395}$-'3	606	58/60
GRIN1 NM_000832.6	5'-$_{1495}$AAGCCTCGAGGGTACCAGAT$_{1511}$-'3 5'-$_{1708}$TGCAAAAGCCGTAGCAACAC$_{1689}$-'3	217	50, 51.9, 53.8, 58 & 60
GRIN1* NM_000832.6	GGTCTGCAGGCTTCGCTCTAG GGCCCGGTCTTCCAGATGTT	490	52.3 & 54.8
GRIN2A NM_001134407.1	5'-$_{1123}$ GACAACAGCTTTGTGGGCTG$_{1141}$-'3 5'-$_{1337}$ TTGGGATGAGCTCCGTGTTC$_{1356}$-'3	235	50, 51.9, 53.8, 58 & 60
GRIN2B* NM_000834.3	5'-$_{271}$ CTCTTCTGAGAACGAGCTCTGCT$_{249}$-'3 5'-$_{777}$ CTCACTTTGTCTGGCCTTGCTTTC$_{753}$-'3	504	52.8 & 54.8
GRIN2C* NM_000835.4	CTCCAAGACCCAAGGCTGCTG ATGTGGCTCTCAGATGCCCTCTAG	510	54.9
GRIN2D NM_000836.2	5'-$_{643}$GTCCTTTGTAGCCGTGACCA$_{662}$-'3 5'-$_{1277}$TCCTGTCTCTGGTGAGGGAG$_{1259}$-'3	635	50, 53.8, 56.1 & 58
GRIN1 NM_000832.6	5'-$_{1495}$AAGCCTCGAGGGTACCAGAT$_{1511}$-'3 5'-$_{1708}$TGCAAAAGCCGTAGCAACAC$_{1689}$-'3	217	50, 51.9, 53.8, 58 & 60
SCN3A NM_006922	5'-$_{3174}$ CATTTTTGCTGTGGTCGGCA $_{3193}$-'3 5'-$_{3957}$ AGCTTGTTCACCTTCTCGGG$_{3938}$-'3	784	54, 56.4/53.1
SCN9A NM_002977.3	5'-$_{635}$ CCGTTTCAATGCCACACCTG$_{645}$-'3 5'-$_{1399}$GCTAAGAAGGCCCAGCTGAA$_{1379}$-'3	758	51.1
SCN11A NM_001287223.1	5'-$_{5824}$GCATCTTTGCCCTGGTAGGT$_{834}$-'3 5'-$_{1466}$GGCTGGTCTTTCCCAGACTC$_{1447}$-'3	643	53.1 & 51.1
CACNA2D1 NM_000722.2	5'-$_{671}$AGGCAGCCAGAGGATAAAACC $_{692}$-'3 5'-$_{991}$GATGTACCATGGTCTTCTGCG $_{970}$-'3	320	51.5/53.3

Table 5.2: Primers, sequences for all investigated receptors and ion channels. Subscript numbering refers to position of the primer on the gene. NCBI accession numbers included. * refers to commercial primers.

5.2 The results of genetic investigation

Using the primers in Table 5.2, human gingival tissue and appropriate cell line (SH-SY5Y) were screened for the presence of the selected gene receptors by PCR at the mRNA level, results are discussed for each gene below.

5.2.1 Adenosine receptors

The expression of adenosine receptor, ADORA2A, at the mRNA level was investigated in the RNA extracted from human gingival tissue and SH-SY5Y cell line. Primers specific for adenosine A2A receptor were designed using NCBI Blast package, Table 5.2. A standard PCR (Section 2.14) was carried out at different annealing temperatures.

Gel electrophoresis showed distinct bands representing PCR products of the receptors generated from both the gingival and SH-SY5Y samples of RNA, Figure 5.1. The PCR products identities were confirmed by sequencing services (Eurofins Genomics, UK) as Adenosine receptor A2A (ADORA2A, NM_001278497.1).

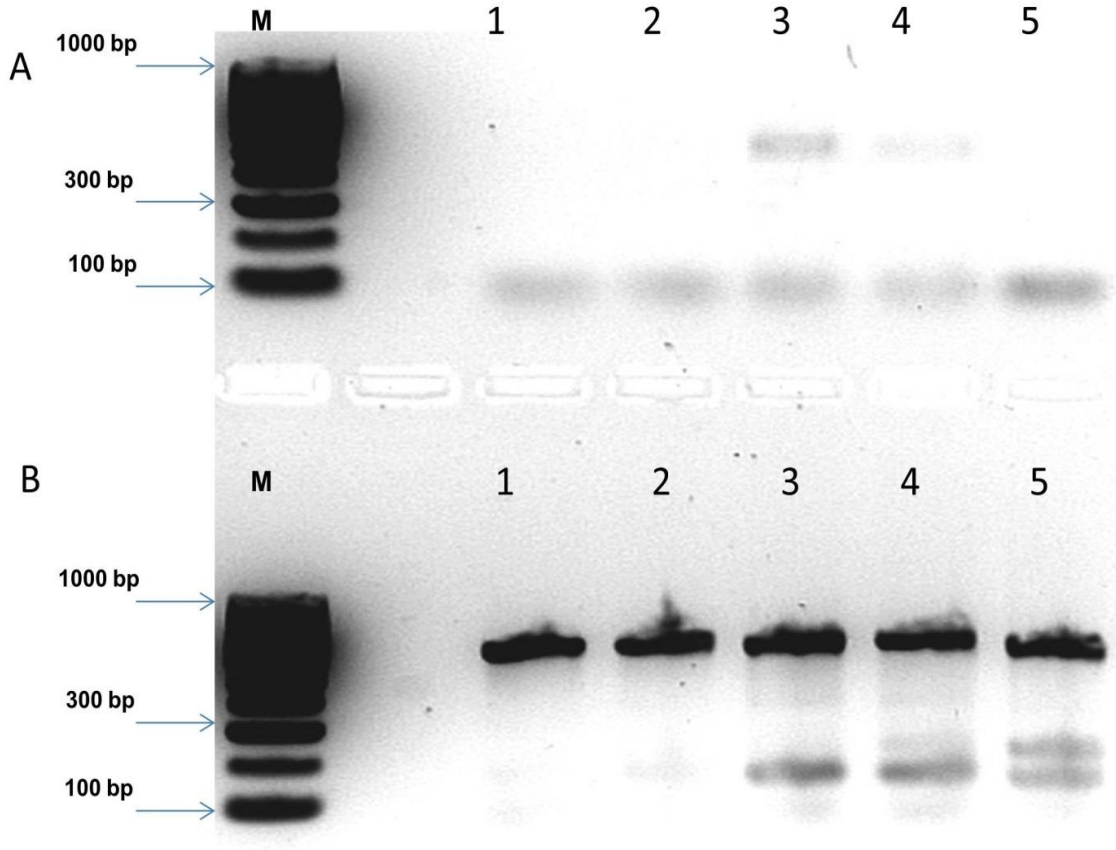


Figure 5.1: PCR products showing the presences of adenosine receptor (ADORA2A) in cDNA prepared from (A) Gingival tissue samples was observed at (58 and 60) °C only (lane 3&4), product was weakly expressed. (B) SH-SY5Y samples at a range of annealing temperatures investigated (50, 51.9, 53.8, 58 and 60) °C shown in lane 1 to 5. In M, hyper ladder IV (Bioline, UK), sizes indicated.

5.2.2 N-methyl D-aspartate (NMDA) channel

The NMDA ionotropic channel is the main ionotropic glutamate receptor. The NMDA1 and NMDA2 (A, B, C & D) subunits of NMDA channel were investigated as the most related NMDA channel subunits to peripheral analgesics in trigeminal area (Marvizon *et al.*, 2002; Cairns, 2009a). The expression of NMDA1 subunit (GRIN1) and four types of NMDA2 subunit (GRIN2A, GRIN2B, GRIN2C and GRIN2D,Table 5.2) were investigated in the RNA extracted from gingival tissue and SH-SY5Y cell line. As cDNA was prepared from the gingival sample was at lower concentration than that from SH-SY5Y, thus further primers were purchased for GRIN1, GRIN2B, GRIN2C and GRIN2D from life technologies.

GRIN1

The primer designed in–house found to be useful in the SH-SY5Y sample only, Figure 5.2. Using the commercial primer revealed distinct bands in the gingival sample and weak expression in SH-SY5Y sample using standard gel electrophoresis procedure, (Figure 5.3). PCR products were confirmed by sequencing services (Eurofins Genomics, UK) for all results as GRIN1 (NM_000832.6).

GRIN2A

Primer specific for NMDA subunit type 2A (GRIN2A) was designed and used in a standard RT-PCR procedure to prepare the cDNA for gel electrophoresis.

A standard gel electrophoresis procedure showed distinct bands in gingival tissue sample only, without expression in the RNA from SH-SY5Y sample, Figure 5.4. The PCR products were further confirmed by sequencing services (Eurofins Genomics, UK) as GRIN2A (NM_001134407.1).

GRIN2B

The primer for NMDA subunit type 2B (GRIN2B) was purchased from Life Technologies Ltd, UK; Table 5.2. A standard PCR procedure was used to prepare the cDNA for gel electrophoresis.

The results of gel electrophoresis revealed expression of a distinct band in the gingival tissue sample only, Figure 5.5, and the results were further confirmed by Eurofins sequencing services as GRIN2B (NM_0.000834).

GRIN2C

The primer for NMDA subunit type 2C (GRIN2C) was purchased from Life Technologies Ltd, UK. A standard PCR procedure was used to prepare the cDNA for gel electrophoresis.

Gel electrophoresis results identify band with gingival tissue sample only, Figure 5.6. The result was confirmed as GRIN2C (NM_000835.4) using Eurofins sequencing service (Eurofins Genomics, UK).

GRIN2D

The primer for NMDA subunit type 2D (GRIN2D) was designed in-house and a cDNA was prepared using standard PCR technique.

Gel electrophoresis results showed weak bands in SH-SY5Y samples only (Figure 5.7) and the results were confirmed by Eurofins sequencing services as GRIN2D (NM_000836.2).

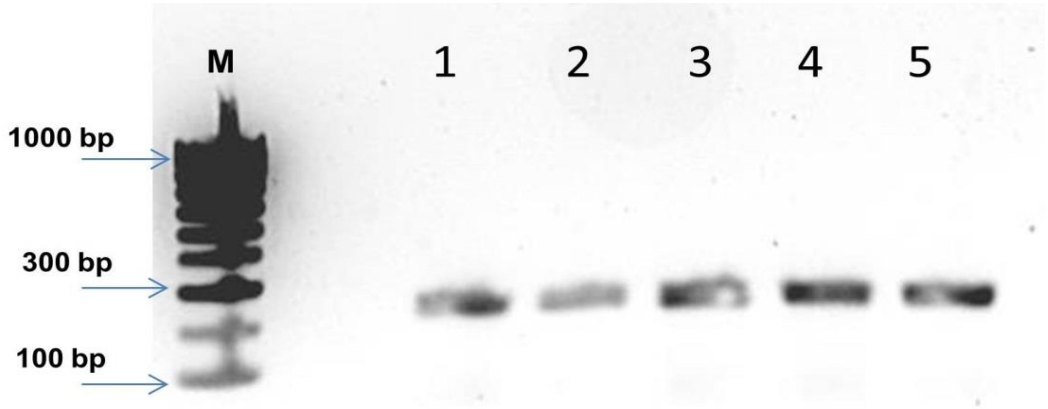


Figure 5.2: PCR products showing the presences of NMDA1 subunit (GRIN1) in cDNA prepared from SH-SY5Y samples at all annealing temperatures investigated (50, 51.9, 53.8, 58 and 60 °C) shown in lane 1 to 5. In M, hyper ladder IV (Bioline, UK), sizes indicated. No expression was detected in gingival tissue sample.

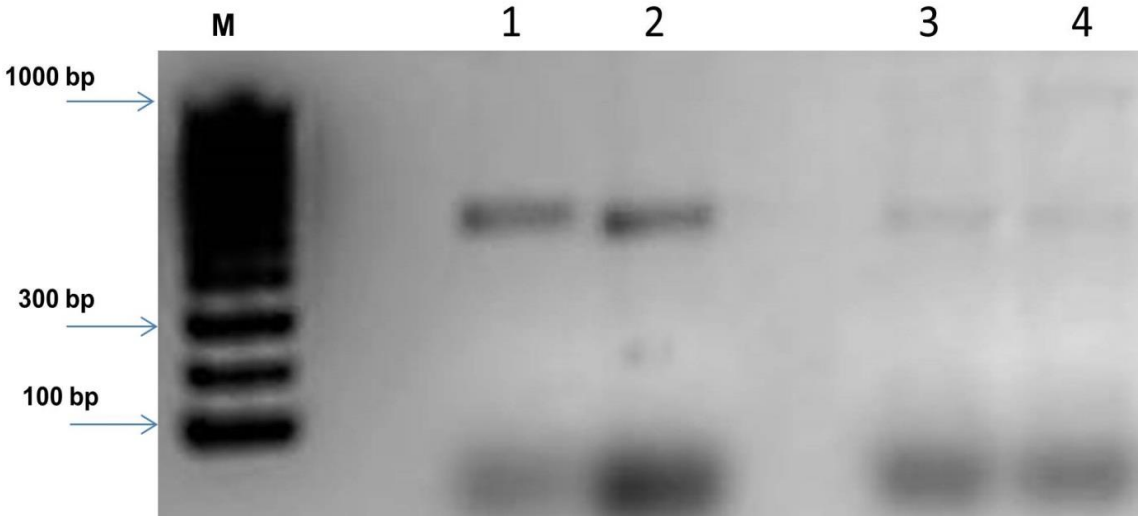


Figure 5.3: PCR products showing the presences of NMDA1 subunit (GRIN1) using commercial primer in cDNA prepared from human gingival tissue (lane 1: 52.3 °C, lane 2: 54. 8 °C) and SH-SY5Y cell line (lane 3: 52.3 °C, lane 4: 54.8 °C). In M, hyper ladder IV (Bioline, UK), sizes indicated.

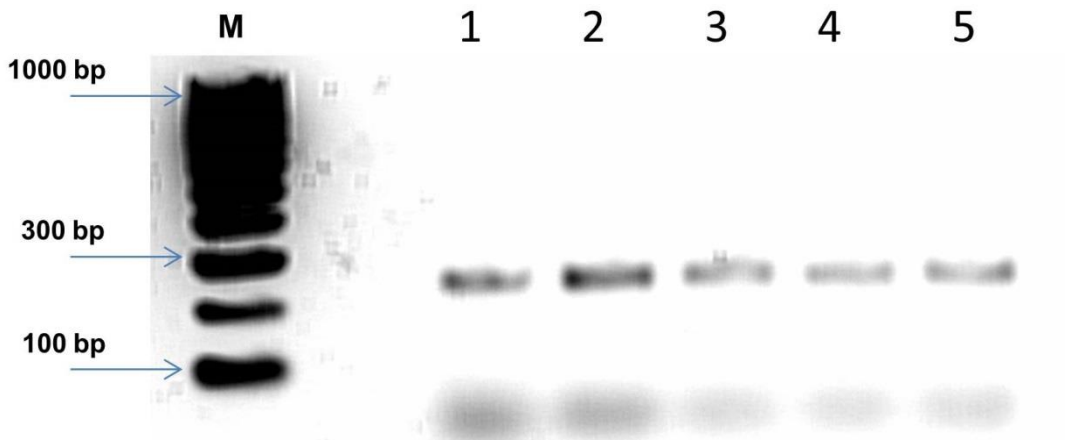


Figure 5.4: PCR products showing the presences of NMDA 2A subunit (GRIN2A) in cDNA prepared from gingival tissue samples only at all annealing temperatures investigated (50, 51.9, 53.8, 58 and 60) °C shown in lane 1 to 5. In M, hyper ladder IV (Bioline, UK), sizes indicated.

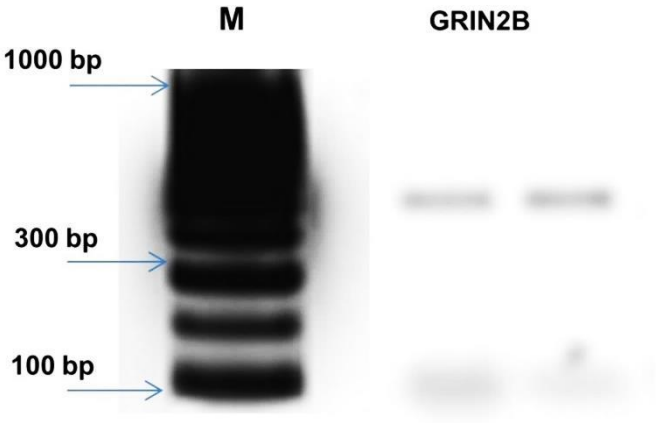


Figure 5.5: PCR products showing the presences of NMDA 2B subunit (GRIN2B) in cDNA prepared from gingival tissue samples only at annealing temperatures investigated (52.8 & 54.8) °C. In M, hyper ladder IV (Bioline, UK), sizes indicated.

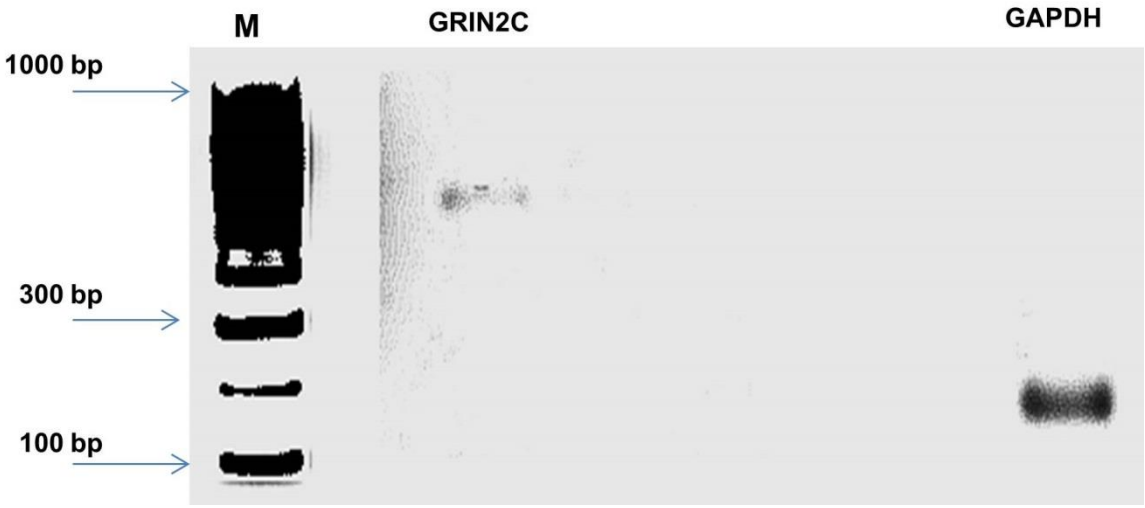


Figure 5.6: PCR products showing weak expression of NMDA2 C subunit (GRIN2C, NM_000835.4) in cDNA prepared from gingival tissue samples only at annealing temperature investigated (54.9) °C. Housekeeping gene (GAPDH) observed at annealing temperature (58) °C. In M, hyper ladder IV (Bioline, UK), sizes indicated.

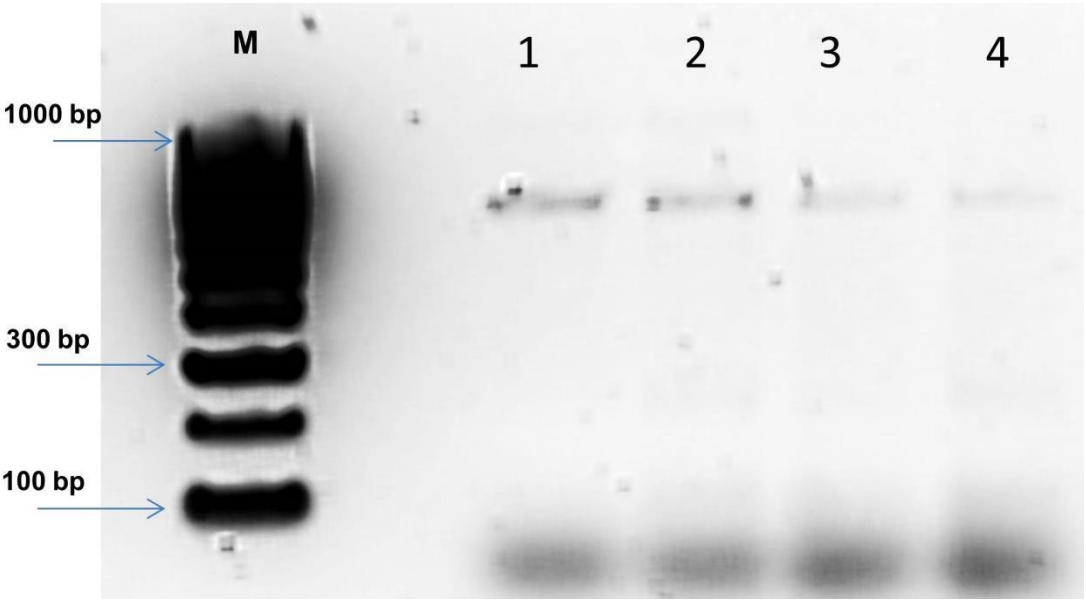


Figure 5.7: PCR products showing weak expression of NMDA2 D subunit (GRIN2D, NM_000836.2) in cDNA prepared from SH-SY5Y cell line samples only at annealing temperature investigated (50, 53.8, 56.1 & 58) °C shown in lane 1 to 4. M, hyper ladder IV (Bioline, UK), sizes indicated. No expression was detected in gingival tissue sample.

5.2.3 Sodium channels

Sodium channels are the main peripheral ion channel involved in pain transduction and are therefore a highly desirable target for topical NMs such as amitriptyline and carbamazepine in order to produce an analgesic effect. Among nine recognised types of sodium channels (Na_v 1.1 to Na_v 1.9), four only have been found to be connected to peripheral pain pathophysiology (Na_v 1.3, Na_v 1.7 to Na_v 1.9), (Cummins, 2009). The investigated sodium channels in this study were: Na_v 1.3, Na_v 1.7 and Na_v 1.9. The primers were prepared using NCBI Blast package (Section 2.14.4), the resultant sequence, product size and annealing temperature are shown in Table 5.2.

The expression of the Na_v 1.3, 1.7 and 1.9 sodium channels (SCN3A, SCN9A and SCN11A respectively) were investigated in gingival tissue and SH-SY5Y RNA samples. Gel electrophoresis results showed distinct bands in both RNA extracted from gingival tissue (Figure 5.8) and SH-SY5Y cell line (Figure 5.9) for SCN3A products and the results identity were identified by Eurofins sequencing service (Eurofins Genomics, UK) as SCN3A (NM_006922). SCN9A was only expressed in SH-SY5Y sample (Figure 5.9) and for SCN11A, the expression at very low level was detected in SH-SY5Y samples only. Both results were identified as SCN9A (NM_002976.3) and SCN11A (NM_002976.3) using sequencing service.

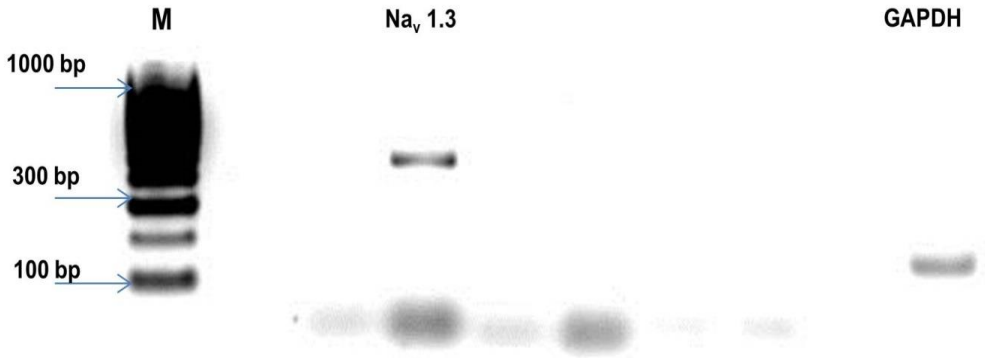


Figure 5.8: PCR products showing the expression of Na_v 1.3 sodium channel (SCN3A) in cDNA prepared from gingival tissue samples at annealing temperature investigated (54) °C. The housekeeping gene (GAPDH) expressed at annealing temperature (58) °C. In M, hyper ladder IV (Bioline, UK), sizes indicated.

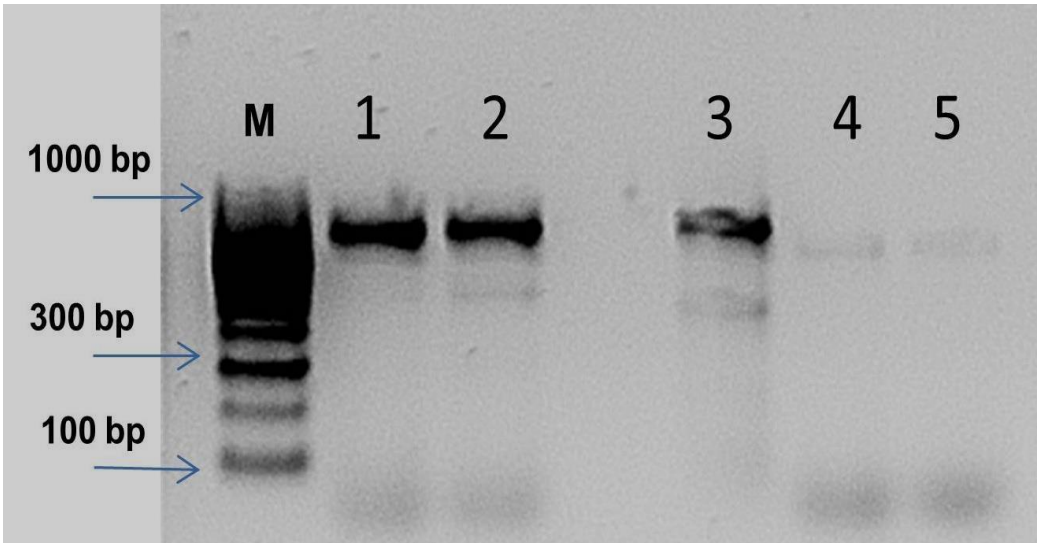


Figure 5.9: PCR products in cDNA prepared from SH-SY5Y samples showing the expression of Na_v 1.3 (SCN3A) at annealing temperatures (56.4/53.1) °C shown in lane 1&2, Na_v 1.7 (SCN9A) at annealing temperature (51.1) °C shown in lane 3 and weak expression of Na_v 1.9 (SCN11A) at annealing temperatures (53.1/51.1) °C shown in lane 4&5. In M, hyper ladder IV (Bioline, UK), sizes indicated.

5.2.4 Calcium channel

The analgesic action of the anticonvulsant gabapentin is ligand to the blocking of calcium channel subunit α2δ-1. The primer of the calcium channel auxiliary subunit α2δ-1 (CACNA2D1) was designed in-house, Table 5.2. A standard PCR procedure was used to prepare the cDNA for gel electrophoresis.

The gel electrophoresis results showed distinct bands in both the RNA from gingival tissue samples and SH-SY5Y samples, Figure 5.10. The results were confirmed by sequencing services as CACNA2D1 (NM_000722.2).

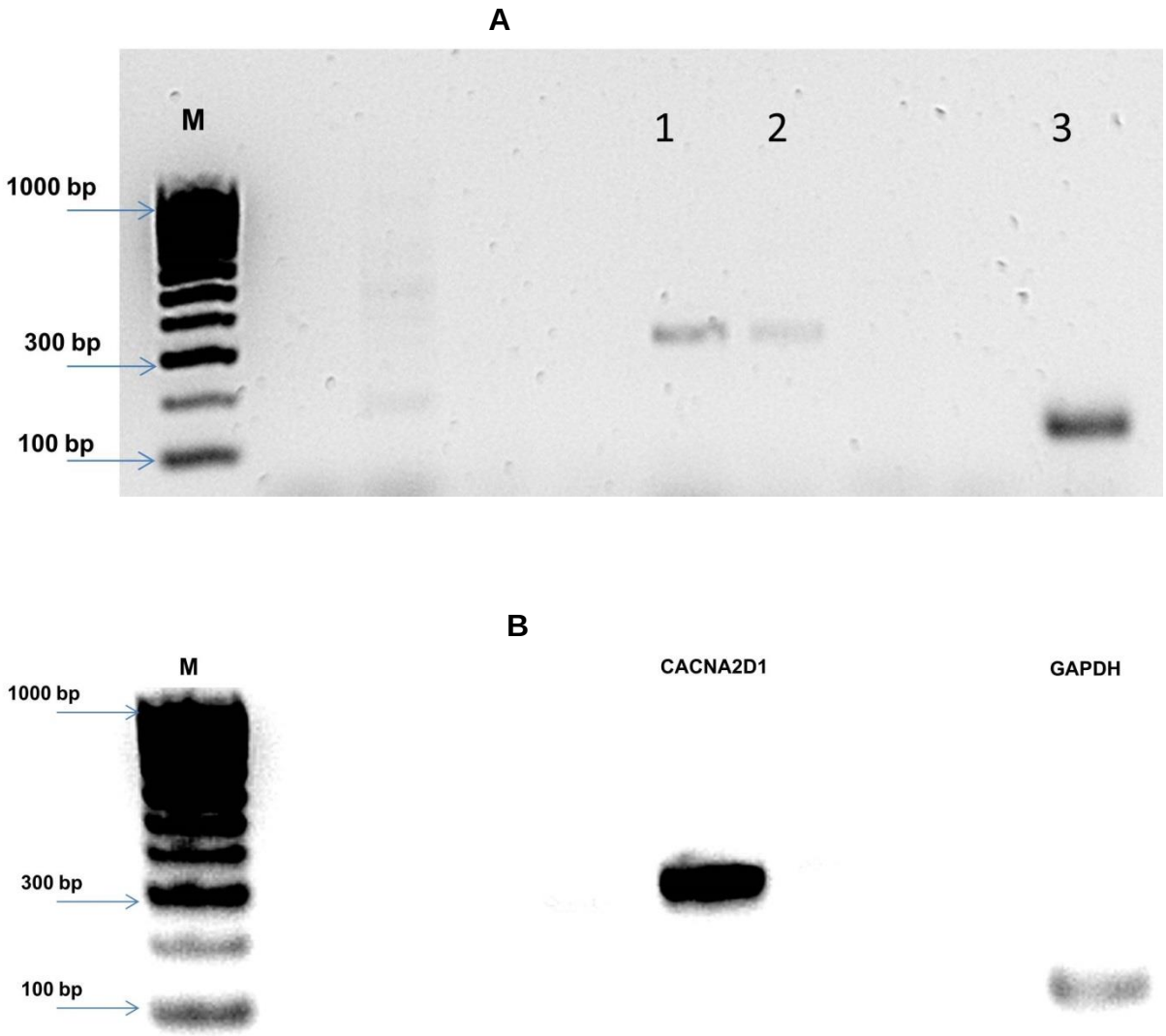


Figure 5.10: PCR products showing the expression of Ca α2δ-1 (CACNA2D1) in cDNA prepared from (A) Gingival tissue samples at annealing temperatures investigated (51.5 & 53.3) °C. The Housekeeping gene (GAPDH) expressed at annealing temperature (58) °C shown in lane 3. (B) SH-SY5Y samples at annealing temperatures (51.5) °C. The Housekeeping gene (GAPDH) expressed at annealing temperature (58) °C. In M, hyper ladder IV (Bioline, UK), sizes indicated

5.3 Semi-quantification of receptors expressed in human gingival RNA

The expression level of genes found to be expressed in the gingival tissue samples were semi-quantitatively assessed in relation to the housekeeping gene (GAPDH) expression in gingival tissue RNA. Although these results are from one biological sample, for semi-quantities analysis, three technical replicates were analysed using gene tools software (SYNGENE, Frederik, USA). The results showed a high level of expression of the glutamate receptor NMDA2A (GRIN2A) and the lower levels of expression for NMDA1 (GRIN1) Figure 5.11, but with wide standard errors.

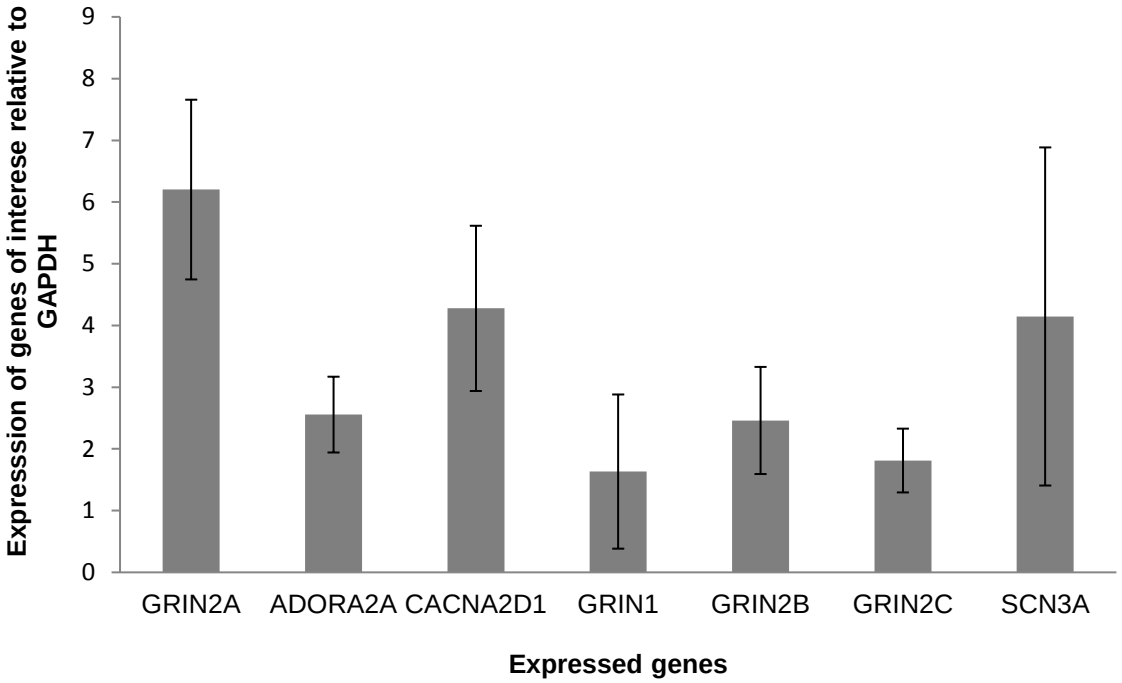


Figure 5.11: Relative expression level of receptors (ADORA2A, GRIN1, GRIN2A, GRIN2B, GTGRIN2C, SCN3A and CACNA2D1) in gingival tissue sample results to the housekeeping gene (GAPDH) expression in the same sample using gene tools software (SYNGENE, Frederik, USA), (SE± mean, n=3 replicate).

5.4 Expression of receptors identified in human gingival tissue RNA in oral keratinocyte cells

The set of genes found to be expressed in gingival tissue sample were also investigated at the mRNA level in oral keratinocyte cells (OKF6-TERT1), (Table 5.3). The gel electrophoresis results revealed the expression of the NMDA subunits genes (GRIN1, GRIN2A, GRIN2B & GRIN2C) in the OKF6-TERT1 sample, but no expression of ADORA2A, SCN3A, and CACNA2D1 receptors were identified, Figure 5.12. The results were confirmed by sequencing service.

Investigated genes	**Human gingival tissue**	**Neuroblastoma cell line (SH-SY5Y)**	**Oral keratinocyte cell line (OKF6-TERT1)**
ADORA2A	×	×	
GRIN1	×	×	×
GRIN2A	×		×
GRIN2B	×		×
GRIN2C	×		×
GRIN2D		×	Not tested
SCN3A	×	×	
SCN9A		×	Not tested
SCN11A		×	Not tested
CACNA2D1	×	×	

Table 5.3: List of the all investigated and expressed genes in the three samples: Human gingival tissue, SH-SY5Y neuroblastoma cell line and OKF6-TER1 oral keratinocyte cell line.

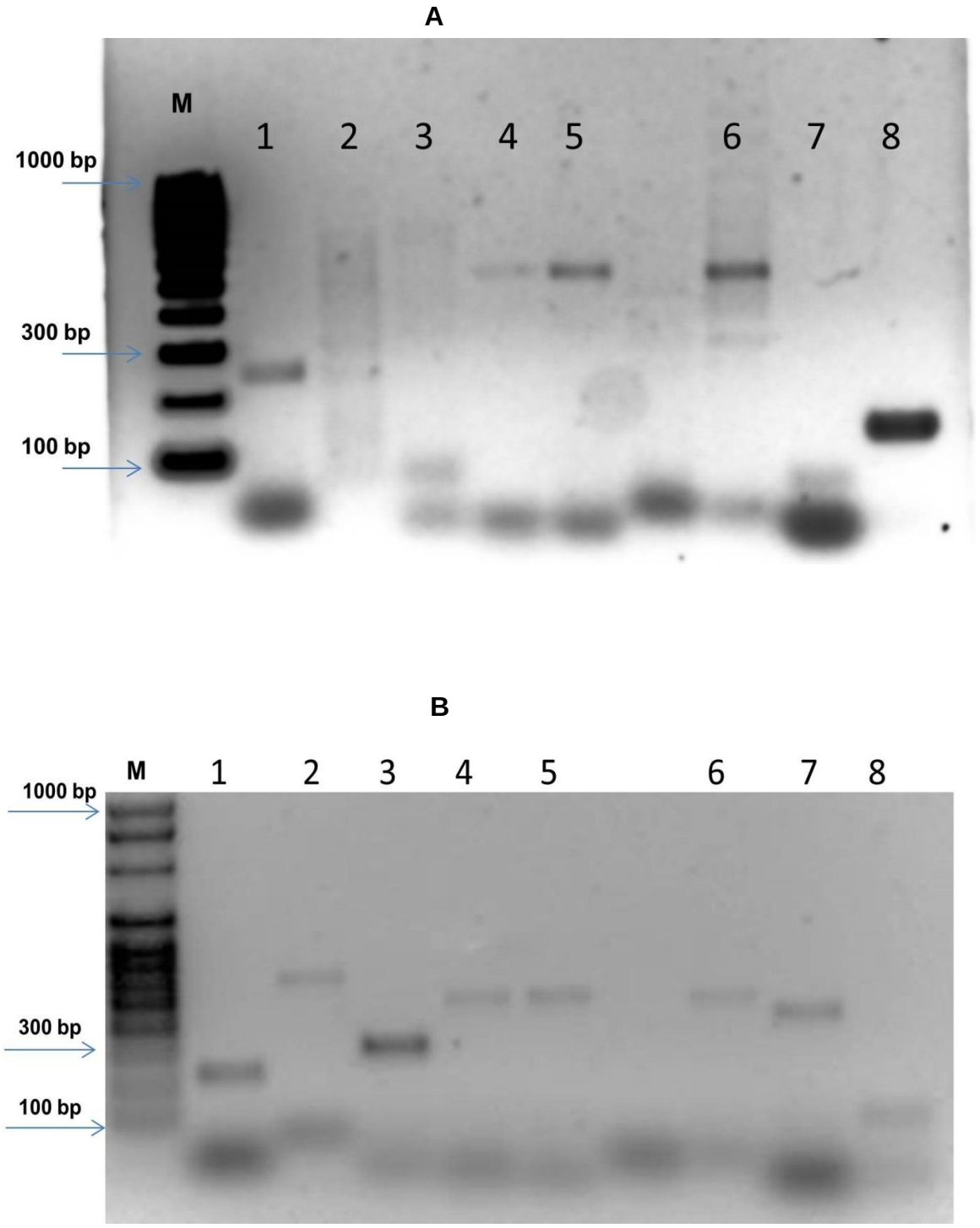


Figure 5.12: PCR products showing the expression of the all investigated genes in (A): cDNA prepared from OKF6-TER1 cell line, at annealing temperatures investigated 53.4 °C for: 1: GRIN2A, 4: GRIN1, 5: GRIN2B, 6: GRIN2C. With housekeeping gene expression shown at lane 8 (GAPDH, 58 °C). (B) cDNA prepared from gingival tissue sample, at annealing temperatures investigated 53.4 °C for: 1: GRIN2A, 2: ADORA2A, 3: CAA2D, 4: GRIN1, 5: GRIN2B, 6: GRIN2C, 7: SCM3A, and 8: housekeeping gene (GAPDH) at 58 °C annealing temperature. In M, hyper ladder IV (Bioline, UK), sizes indicated.

5.5 DISCUSSION

The apparent safety of topical treatment along with the concept of the presence of key peripheral nervous system receptors has escalated the prescription and compounding of the topical neuromodulatory treatments (Sawynok, 2003; Baron, 2006; Mick *et al.*, 2011). The crucial role of peripheral receptors and ion channels in the pathophysiology of neuropathic pain have been investigated and reported in many *in vitro,* animal and human functional studies (Hirai and Hama, 2014). However, these studies have their limitations for example: these studies used an acute model of pain as a representative model of chronic *in vivo* human conditions; and in functional studies there is great subjectivity (Blackburn-Munro, 2004; Mogil, 2009). This results in knowledge shortages and necessitate more advance human based studies to fulfil the need of a greater understanding of neuropathic pain pathophysiology, for better treatment choices (Micó *et al.*, 2006).

This study was designed as a preliminary investigation into the types of peripheral receptor expressed in the human gingival tissue, as a human tissue that is likely to be representative of peripheral trigeminal sensory innervation. These preliminary results should help in designing longer and more thorough molecular studies, investigating the expression and the role of these receptors in NOP and the most suitable topical medication for the management of PNP.

The selection of receptors was based upon the three NMs (amitriptyline, carbamazepine and gabapentin) used and their peripheral analgesic pathway. This was a targeted approach, using only one human gingival sample, and not all receptors and ion channels were represented.

The Adenosine receptors are expected to be a typical target for the peripheral acting NMs, such as amitriptyline and carbamazepine (Sawynok *et al.*, 1999; Sawynok *et al.*, 2005; Micó *et al.*, 2006; Lee and Abrahams, 2008). Two types of adenosine receptors (A1, A2A) are suggested to be expressed peripherally on both type of nerve endings: the myelinated (Aδ–fibers) and unmyelinated (C-fibers) (Sawynok, 2009). This genetic expression investigation showed the expression of A2A receptor (ADORA2A) at mRNA level in both gingival tissue

and SH-SY5Y samples. However, the investigations did not reveal adenosine receptor A1 expression in both gingival tissue and (SH-SY5Y) samples. The results of this exploring investigation indicate that ADORA2A might be involved in adenosine mediated analgesic process induced by NMs topical action.

The prevention of glutamate action, the main excitatory amino acid, would induce analgesic action in the target site; the limitations of glutamate receptor (NMDA channel) antagonists did not limit the clinical and animal trials using different medications such as amitriptyline (Ushida *et al.*, 2002; Lynch *et al.*, 2005b; Cairns, 2009a; Mercadante, 2015). The expression of NMDA ionotropic channel subunits (NMDA 1, NMDA 2 A, B, C& D) subunits was investigated in both the gingival tissue and SH-SY5Y samples. With exception of the NMDA2D (GRIN2D) subunit, the results showed the expression of three subunits (GRIN2A, GRIN2B & GRIN2C) in gingival tissue samples only, while NMDA1 (GRIN1) was expressed in both. The expression of NMDA receptor subunits may justify the topical analgesic action of NMs such as amitriptyline and carbamazepine.

Sodium channels are typical targets for the peripheral analgesic agents such as local anaesthetics, in addition to the affinity of tricyclic antidepressants (amitriptyline) and anticonvulsants (carbamazepine) for sodium channels. The levels of expression of sodium channels investigated in this Chapter (Na_v 1.3, 7 & 9) were relatively low or not detected in the gingival tissue sample. Their expression might have been affected by the local anaesthetics which was used in the surgical excision procedure to obtain the sample. Conversely, in SH-SY5Y samples, the levels of expression of all the sodium channels were relatively high. This area needs further examination and it may be possible to obtain samples where general, rather than local, anaesthesia has been used thereby removing the potential confounding influence of local anaesthesia. Our preliminary results for receptor expression should therefore be subject cautious interpretation and require further confirmation from a wider selection and number of samples including unattached and attached gingiva from elsewhere in the oral cavity.

The increased evidence of correlation between expression and upregulation of the calcium channel α2δ-1 subunit during neuropathic pain, makes it as a good target for the peripheral analgesic effect (Maneuf *et al.*, 2006; Li *et al.*, 2014). Anticonvulsants such as gabapentin have affinity for calcium channel α2δ-1 subunit, inducing the analgesic by blocking pain perception, propagation and decreasing neurotransmitter release (McGivern, 2009). The gel electrophoresis and gene sequencing showed the expression of the calcium channel α2δ-1 (CACNA2D1) subunit in gingival tissue as well as cell line sample (SH-SY5Y), indicating this could be the site of action for gabapentin topical treatments.

The semi-quantitative assessment of the expressed genes comparing to the house keeping gene (GAPDH) expression, showed the higher expression was belong to the glutamate receptor subunit (GRIN2A). This is the results of one sample, it could be a recognised possibility, but further work may be by including more samples and/or growing cells *in vitro* to measure how the expression of these receptors would be affected by topical treatment exposure.

The full thickness gingival tissue sample is not merely representative of trigeminal nerve ending, as the main histological component is epithelial keratinocyte cells. To identify if there is a cross expression in non-neuronal cells such as oral keratinocytes cells, the genes found to be expressed in the gingival biopsy samples were also investigated in the oral keratinocyte cell line (OKF6-TERT1) RNA. The results of gel electrophoresis and sequencing showed the expression of the all glutamate receptor (NMDA) subunit in the OKF6-TERT1 samples (GRIN1, GRIN2A, GRIN2B & GRIN2C). This may indicate that the expressed NMDA receptors are related to the keratinocytes' part of the gingival tissue. The expression of NMDA channel subunits on keratinocyte cell lines were previously reported, however, investigation of the glutamate receptors and all other expressed genes would need more thorough immunohistochemistry studies to recognise the expression site of these receptors and their role as possible targets in peripheral analgesic modality (Skerry and Genever, 2001; Nahm *et al.*, 2004).

The results from one human gingival biopsy using a target gene expression approach, showed the expression of most peripheral targets for topical NMs

analgesics investigated in this study. This was a preliminary study using a human sample with genetic investigation. The results would help in designing a thorough study and employing more human samples. The possibility of employing a patient samples may be limited by the nature of the disease and the difficulty of surgical intervention in patients already suffering with chances of more nerve damage, however, skin biopsy employment is approved in peripheral neuropathy studies with possibility of various investigations to be applied (Cruccu *et al.*, 2010; Brouwer *et al.*, 2015).

Chapter 6: Summary, conclusions and future work

6.1 Summary

The use of topical NMs is off-label treatment; that is compounded and prescribed “outside of the terms of their licence” (GMC, 2015) according to patient needs (FDA, 2015a). The topical NMs are already approved as systemic medications, but no topical route has been licenced. Interest has increased in topical NMs, because they potentially offer relief of NOP with less side effects which hopefully improves patient adherence and satisfaction. This study was designed to investigate the safety of these drugs applied topically modality and the existence of essential receptors that justify their topical application and analgesic action.

In the first group of investigations, the effects of three commonly prescribed NMs (amitriptyline, carbamazepine and gabapentin) were tested on a two-dimensional (2D) monolayer cell culture. Four assays were carried out: cellular viability, count, morphology and inflammatory cytokine expression. The *in vivo* clinical application and regimen for these topical treatments was considered in the design of the monolayer studies. Specifically we sought to emulate the dosing regimen and exposure times likely to be realistic in the clinical application of the drug.

Both amitriptyline concentrations (200 µM, 1.8 mM), caused a significant reduction in the cellular viability and cell counts were less than 10 % of the original seeding count after the long time point exposure (24 h) in the 2D model. Alongside the findings from SEM, which included shrinkage and loss of attachment, these viability and cell count results helped confirmed that amitriptyline was cytotoxic in 2D monolayer cell culture.

The two anti-convulsants (carbamazepine and gabapentin) were less cytotoxic than amitriptyline in the 2D monolayer cell culture. The cytotoxicity and anti-proliferative effects of carbamazepine occurred at high concentrations (1.7 mM) and long exposure time (24 h) with minor morphological changes. Gabapentin appeared to be the least cytotoxic of all three agents and no changes were apparent on SEM either. All three NMs tested appeared not to significantly

affect cytokine expression, but this result may be related to the short exposure time (30 min) and examination method used. The key finding of the monolayer cell culture study is that amitriptyline was cytotoxic even with low concentration and short exposure time. The cells used in this chapter are relevant to the orofacial tissue of interest, however, the *in vivo* environment is more complicated to be fairly represented by monolayer cell culture.

So Despite these seemingly definitive findings it was necessary to examine them further and see if they were validated in a model that was more representative of the in vivo tissue, a 3D tissue model (Maltman and Przyborski, 2010). The cells used in both models are primary cells as in MatTek model and cell line as in in-house constructed model.

The type of 3D tissue culture gave the opportunity to investigate viability and to design more robust and representative experiments as in apoptosis investigation using anti-caspase 3 antibodies and inspect amitriptyline passage through the tissue.

The same viability assay was used in 3D model investigations (alamarBlue®) as in the monolayer cell culture. In addition, however, a different colorimetric assay to measure cytotoxicity parameter (LDH) was also employed. The type of culture in a 3D model (liquid air interface) also gave the opportunity for additional investigations and the employment of different time points (repeated exposure time points: B.I.D. and T.I.D.) in addition to those time points previously tested in the 2D monolayer cell culture (30 min and 24 h). The application of these repeated exposure time points facilitated the examination of any possible reversible toxicity and allowed for a better simulation of the clinical environment (EMA, 2010).

Both colorimetric results in the 3D model showed reduced viability and increased toxicity after amitriptyline exposure. The effect of amitriptyline on the 3D model was further investigated using H&E staining and two types of anti-caspase 3 antibodies to investigate any apoptotic activity of amitriptyline (Green and Reed, 1998; Lirk *et al.*, 2006a; Bautista-Ferrufino *et al.*, 2011). H&E investigations did not demonstrate any gross structural changes following

Amitriptyline exposure. Apoptotic investigations showed that amitriptyline exposure was associated with an increase in apoptotic activity, especially with repeated exposure (B.I.D. and T.I.D.) in MatTek model and long time exposure (24 h) in both MatTek and in-house constructed models.

Despite it was only repeated short exposure (30 min) of low concentration of amitriptyline (226 µM), the apoptosis incidence gave a strong evidence of amitriptyline toxicity, and clinical avoidance is advisable especially with long term clinical application.

Given the expected short (30 min) clinical exposure of topical amitriptyline to oral tissues, its passage through such tissue was also questionable. 3D tissue culture was used to investigate the passage of amitriptyline using chemical analysis (HPLC) and immunofluorescence investigation (anti-amitriptyline anti body). The results of both assays revealed the presence and passage of amitriptyline through tissue after short application time.

The results of 3D model revealed that: amitriptyline has strong apoptotic activity even though only short repeated exposure times were employed; amitriptyline does move through the tissues after short periods of repeated application and this is related to amitriptyline's apoptotic activity and chemical structure. However, the 3D model used in this study is representing oral gingival tissue only, and investigating amitriptyline's apoptotic effect on skin 3D tissue is necessary.

Developing the 3D model and using it in this study gave more representative and robust results regarding *in vivo* environment reproduction. This type of culture will help to decrease expenses of commercial model and could be modified to perform different type of *in vivo* investigations.

The presence of specific peripheral receptors and ion channels, as therapeutic targets for any of the topically applied NMs, is essential for them to exert any non-placebo analgesic mechanism. The goal of using a human gingival biopsy was to investigate the expression of these receptors and ion channels in orofacial tissue. The preliminary genetic investigation in this thesis demonstrated the expression of most of the desired receptors in relation to the

analgesic mechanism of the investigated NMs. In spite of the limitation of a single sample, the preliminary results in this thesis are encouraging and future studies using more sophisticated examination modalities are required to further investigate this.

6.2 Conclusions

This *in vitro* study was designed to define the responses of range of cells and tissue to pharmacological concentrations of a group of medications (NMs) frequently reported to be applied topically for neuropathic pain treatment. The medications are: amitriptyline, carbamazepine and gabapentin.

The first objective of this thesis was to define the effect of the tested NMs on monolayer cell culture viability, count and morphology with inflammatory cytokine profile investigation. The results showed a high toxic effect of amitriptyline on cellular viability, count and morphology in all cell lines.

Carbamazepine showed minor morphological changes. Reduction in viability and count associated mainly with high concentrations of carbamazepine. Gabapentin presented lesser toxic effect without morphological and major cell count negative effects. The inflammatory cytokine expression did not change significantly in response to the exposure of the three NMs.

The second objective was to use 3D model cell culture to investigate the effect of amitriptyline on viability, cytotoxicity, tissue morphological changes and apoptosis activity, in addition to examining the passage of amitriptyline through 3D tissue. The results of viability, cytotoxicity and morphological changes in the 3D model revealed the cytotoxic effect of amitriptyline, but these results were not definitive. The investigation of apoptosis in the 3D model showed a high apoptotic activity through 3D tissue layers in response to repeated exposure of amitriptyline. The results of 3D tissue showed the ability of the topical amitriptyline to infiltrate through tissue barriers.

The final objective was a preliminary genetic study, designed to explore the expression of the peripheral receptors in the orofacial tissue. The results of the genetic investigation revealed the expression of the most investigated genes related to the suggested analgesic pathway of the three NMs.

6.3 Future work

6.3.1 For in vitro studies:

1- Examining the cytotoxicity of more known compounded topical treatments used such as baclofen, clonidine and clonazepam.
2- Using neuronal tissue such as SH-SY5Y to investigate the effect of three NMs on the expression of peripheral receptors.
3- Using the 3D tissue model to study the passage of the NMs that have been identified as less cytotoxic (carbamazepine and gabapentin) in 2D models.
4- Investing more time in constructing a 3D model using human primary cells and implanting a neuron cell within full thickness 3D tissue. This model would help to understand at short term, what types of changes would occur after the application of NMs.
5- Examining the change in inflammatory cytokine profile in response to NMs exposure using a different and more sensitive method such as ELISA.
6- Using skin 3D tissue model to investigate the apoptotic effect of amitriptyline.

6.3.2 For animal studies:

1- Animal models could be used to study the cytotoxicity of low concentration of carbamazepine and amitriptyline, before suggesting a clinical study.
2- Topical application of NMs in animal model will give the opportunity to study the change in expression of peripheral receptors using immunohistochemistry and genetic investigations. NOP might be induced in the animal model to study the differences in expression by using different pain models and application types (including: before and after treatment and control models).

6.3.3 For human studies

Human samples could also be used to investigate the changes in expression and structure of the peripheral receptors in relation to the pathophysiology of NOP, but despite other areas receiving ethical approval (for example dermatological samples for peripheral neuropathy (Cruccu *et al.*, 2010; Nolano *et al.*, 2013)) this raises some difficult ethical problems such as the need for invasive surgery to obtain the samples in patients whose nervous systems are already at least peripherally sensitised. It may be that with advances in less invasive techniques such as brush biopsies and advances in immunohistochemical techniques this may become possible in future years without the need to subject patients to surgery. If advances are made it would offer the opportunity for various investigations and the results are likely to be more representative than animal and *in vitro* findings.

The other possible method of getting human samples is the human post mortem tissue and fluids related to the orofacial pain. These samples would offer an opportunity to investigate the effect of the prescribed treatment on the pathophysiology of NOP especially with the availability of clinical records.

6.3.4 For clinical studies

The results of this *in vitro* study revealed the safety of gabapentin as a topical treatment that may help to design a clinical trial using gabapentin as a compounded topical treatment in management of NOP. The anticonvulsant gabapentin was found useful in management of different NP such as PHN and posttraumatic neuropathic pain. A low cost double blind N-of-1 trial for patient with localised NOP pain could be designed. It is helpful to compare topical gabapentin effect with placebo or other treatment, in which treatment effect could be easily validated by visual analog scale (VAS) or quantitative sensory testing (QST).

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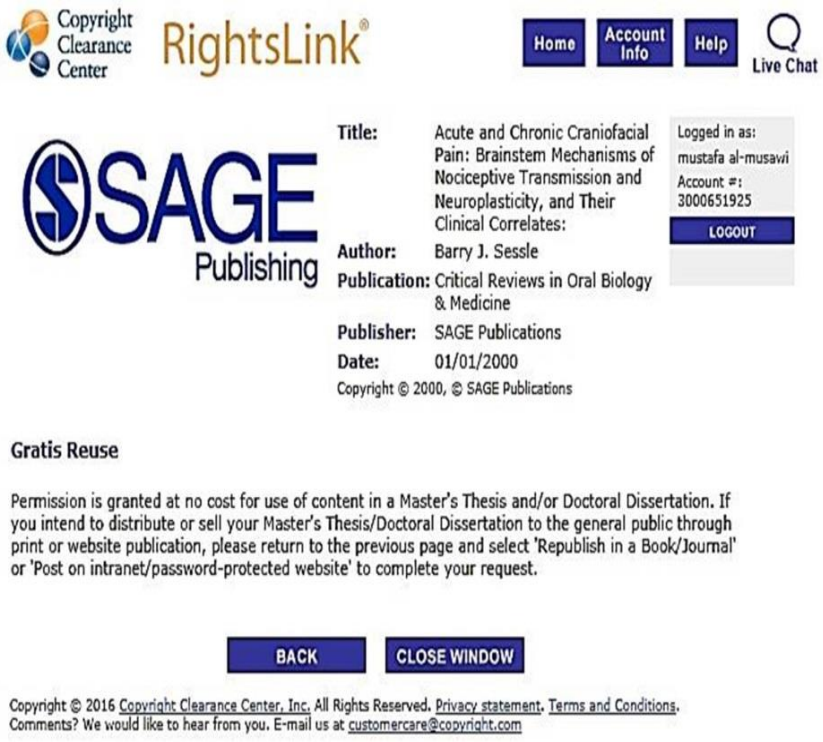

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Appendix B: Cumulative viability results in 3T3, HaCat and OKF6-TERT1 cell lines

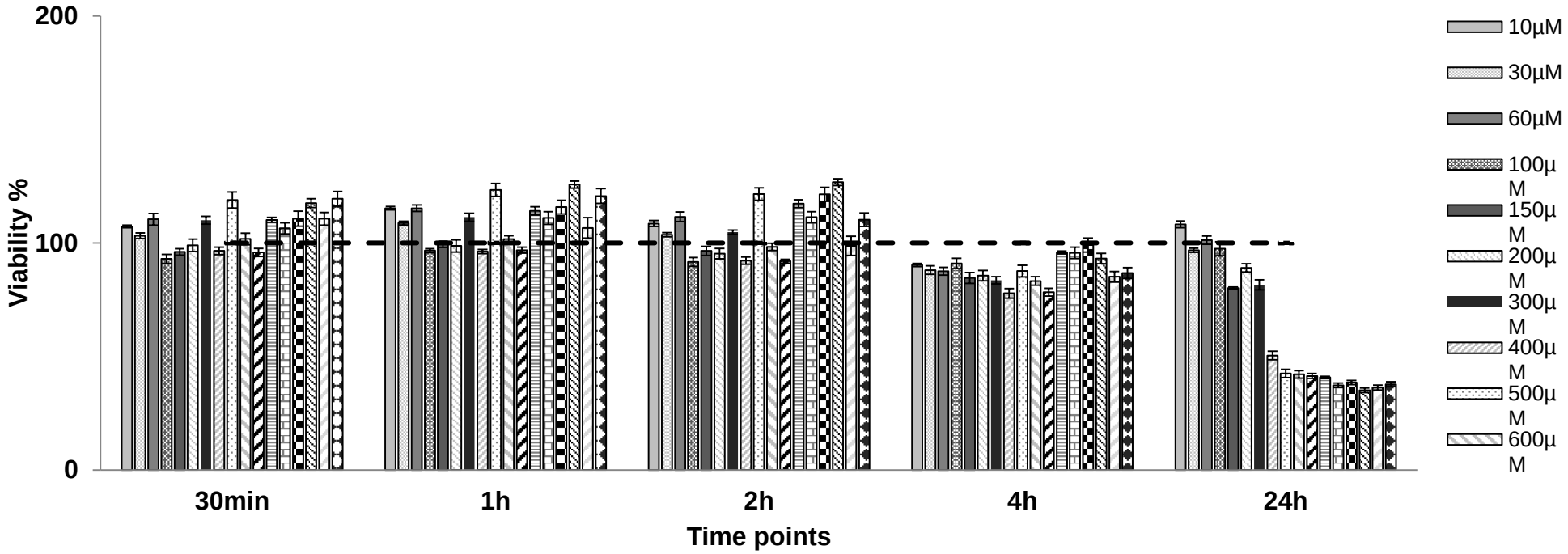

Figure B.1: Cellular viability of HaCat cells exposed to different concentrations of carbamazepine (CBZ), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

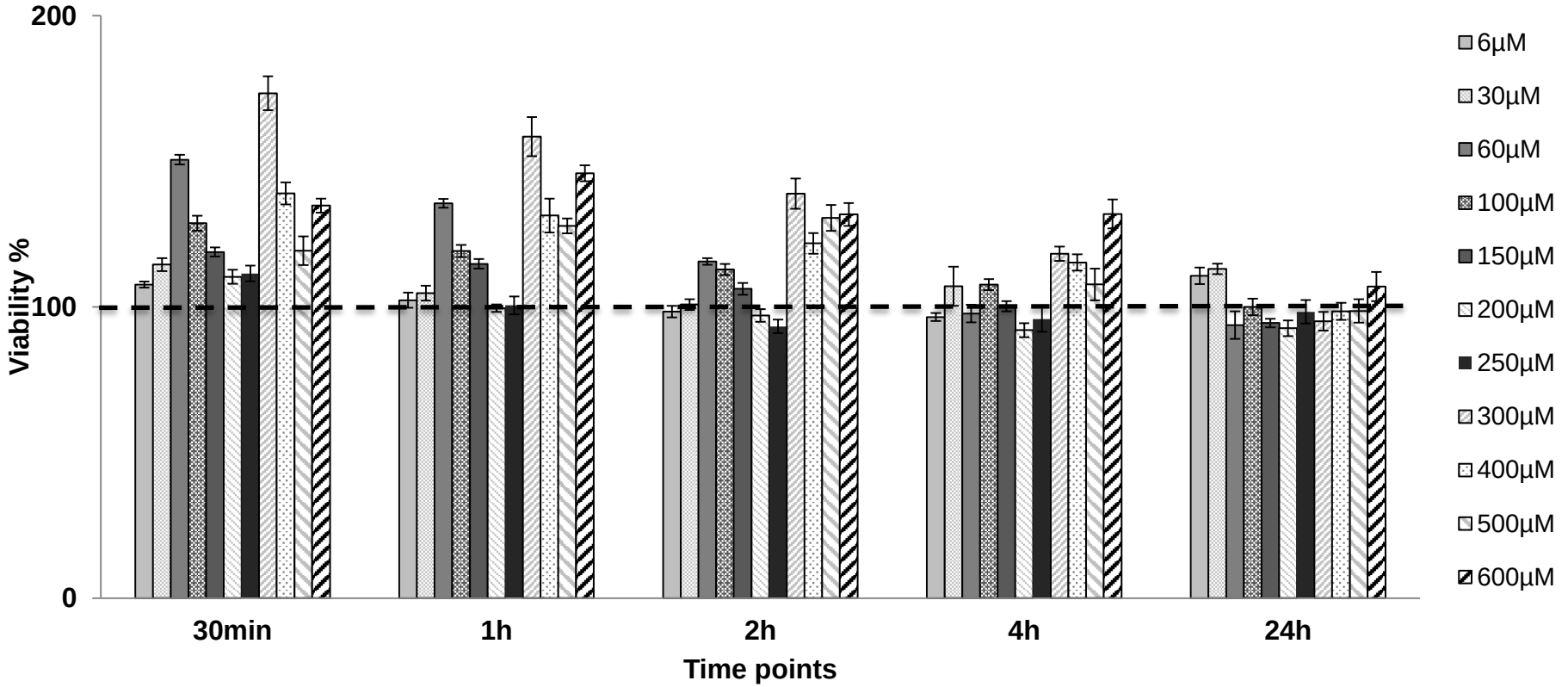

Figure B.2: Cellular viability of HaCat cells exposed to different concentrations of gabapentin (GAB), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

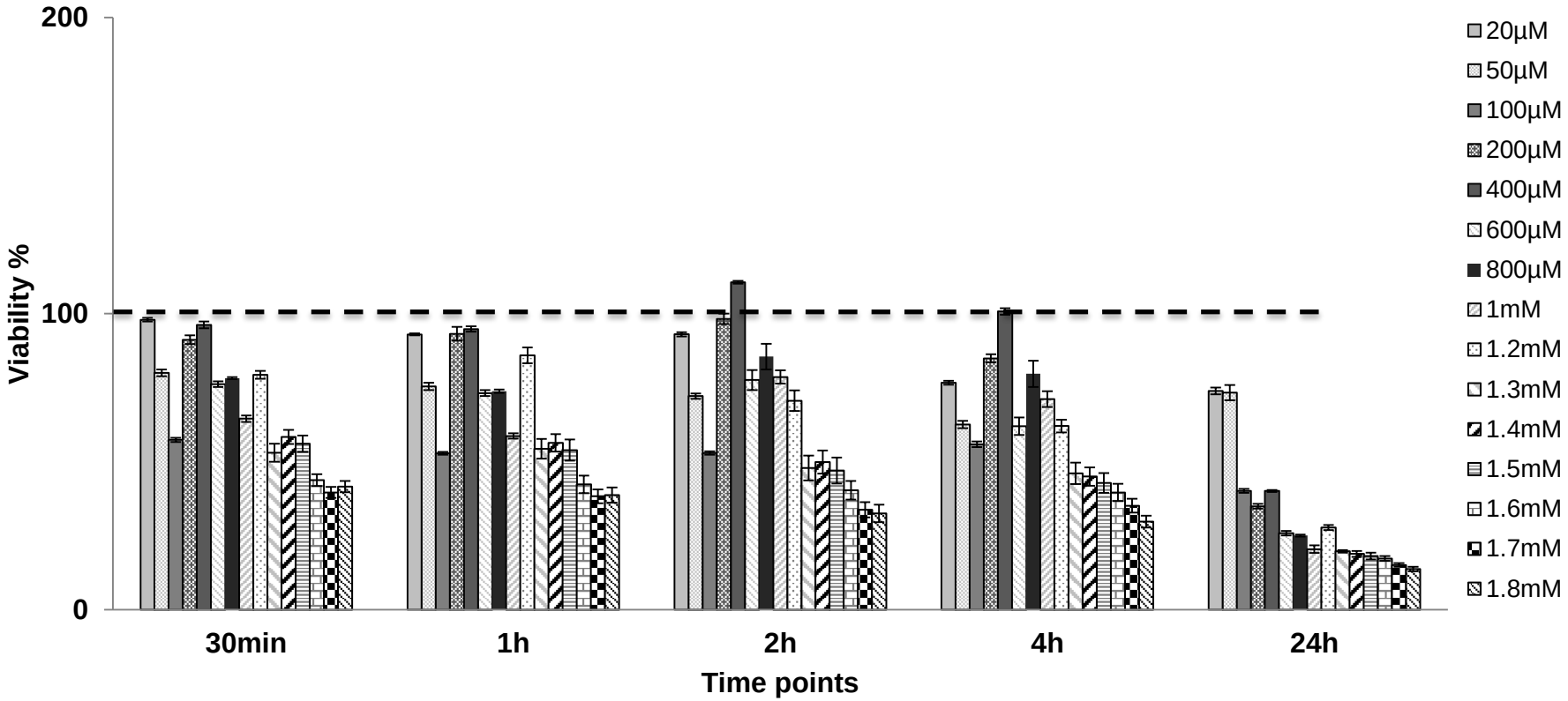

Figure B.3: Cellular viability of HaCat cells exposed to different concentrations of amitriptyline (AMI), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

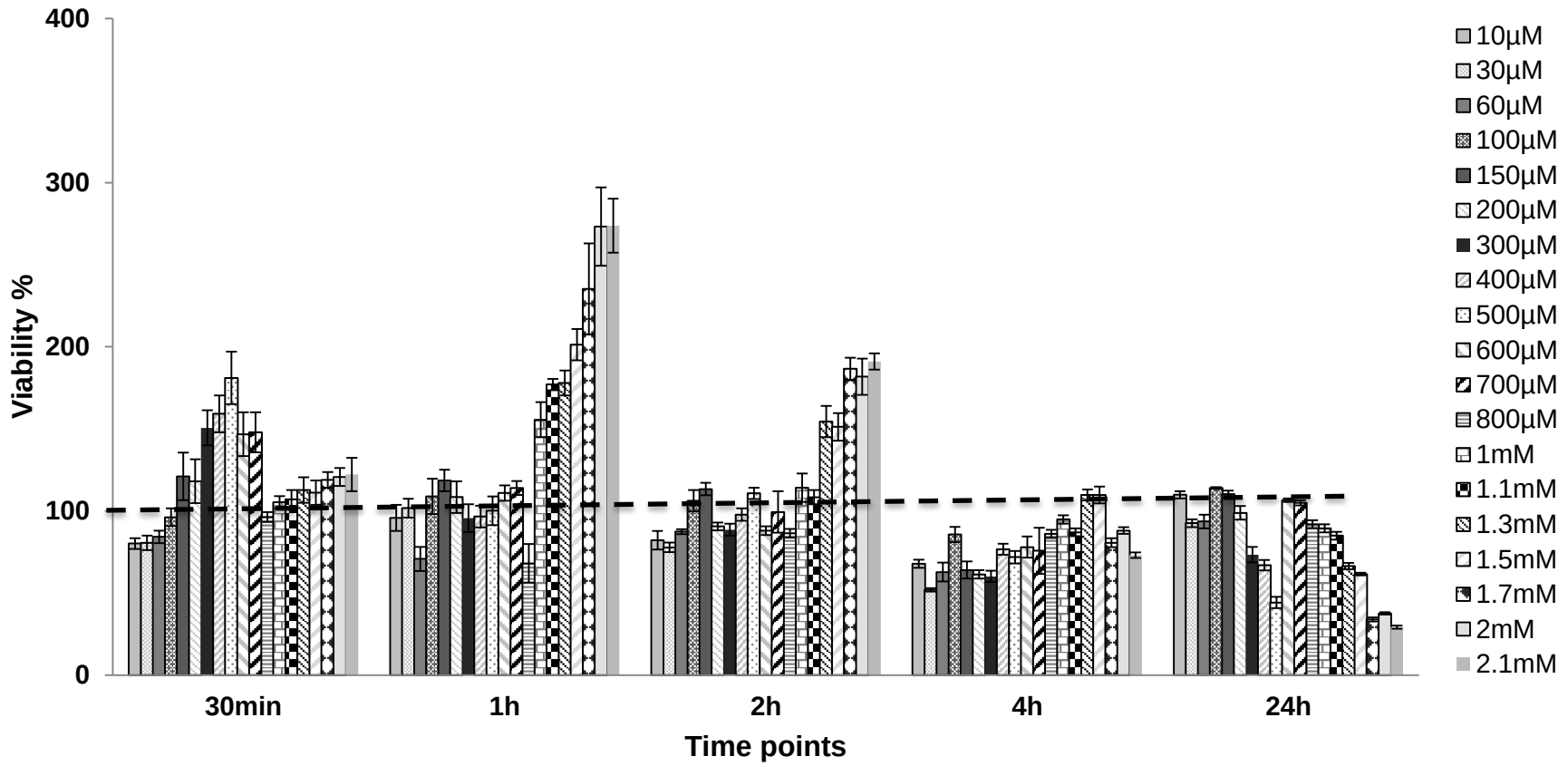

Figure B.4: Cellular viability of OKF6-TERT1 cells exposed to different concentrations of carbamazepine (CBZ), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

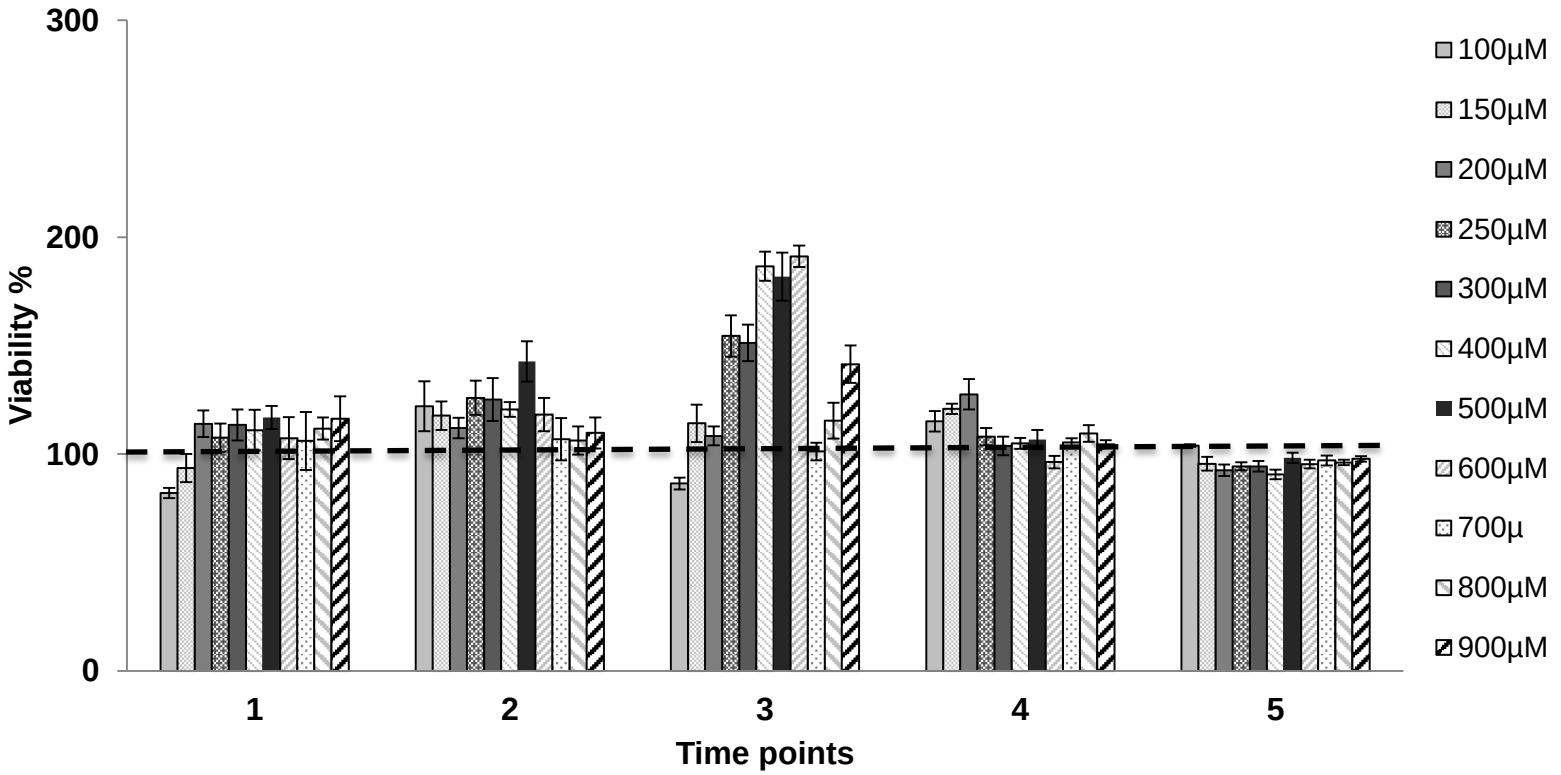

Figure B.5: Cellular viability of OKF6-TERT1 cells exposed to different concentrations of gabapentin (GAB), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

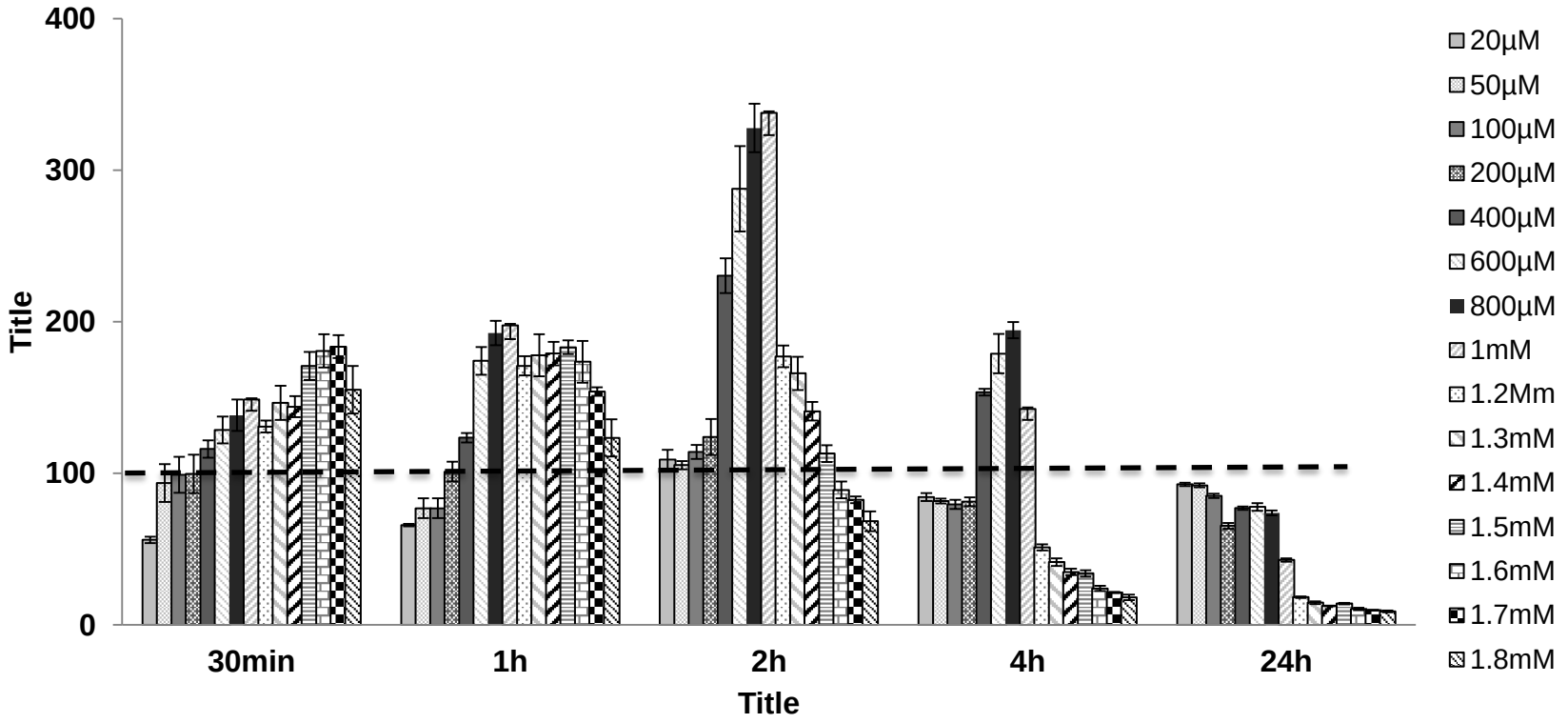

Figure B.6: Cellular viability of OKF6-TERT1 cells exposed to different concentrations of amitriptyline (AMI), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

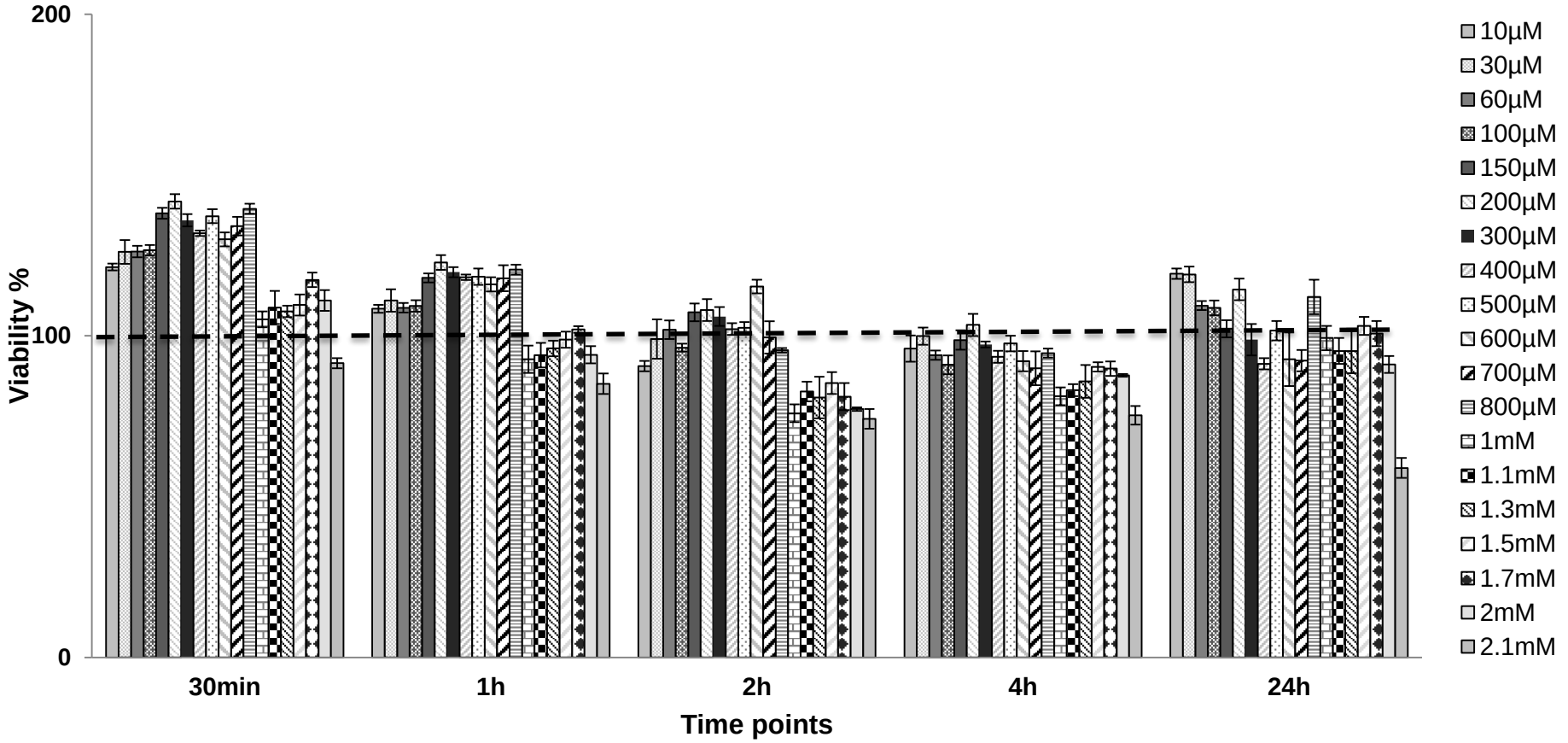

Figure B.7: Cellular viability of 3T3 cells exposed to different concentrations of carbamazepine (CBZ), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

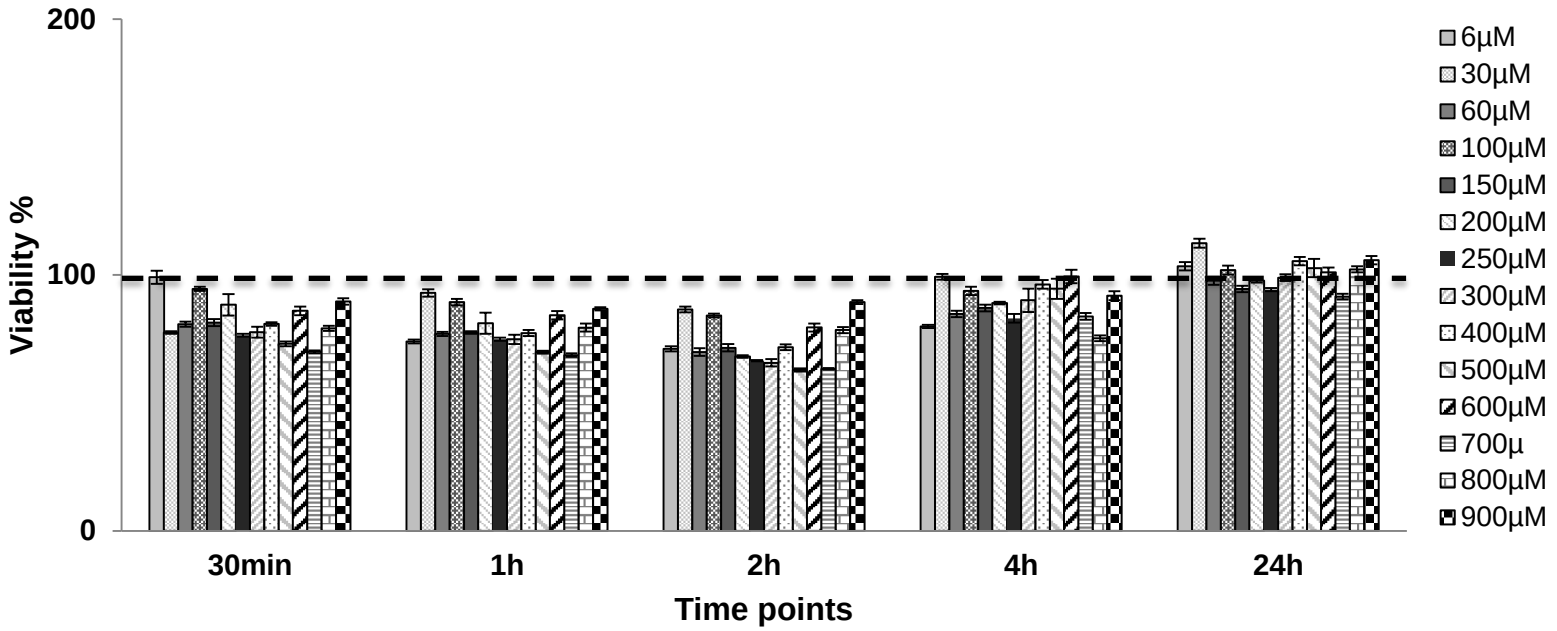

Figure B.8: Cellular viability of 3T3 cells exposed to different concentrations of gabapentin (GAB), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

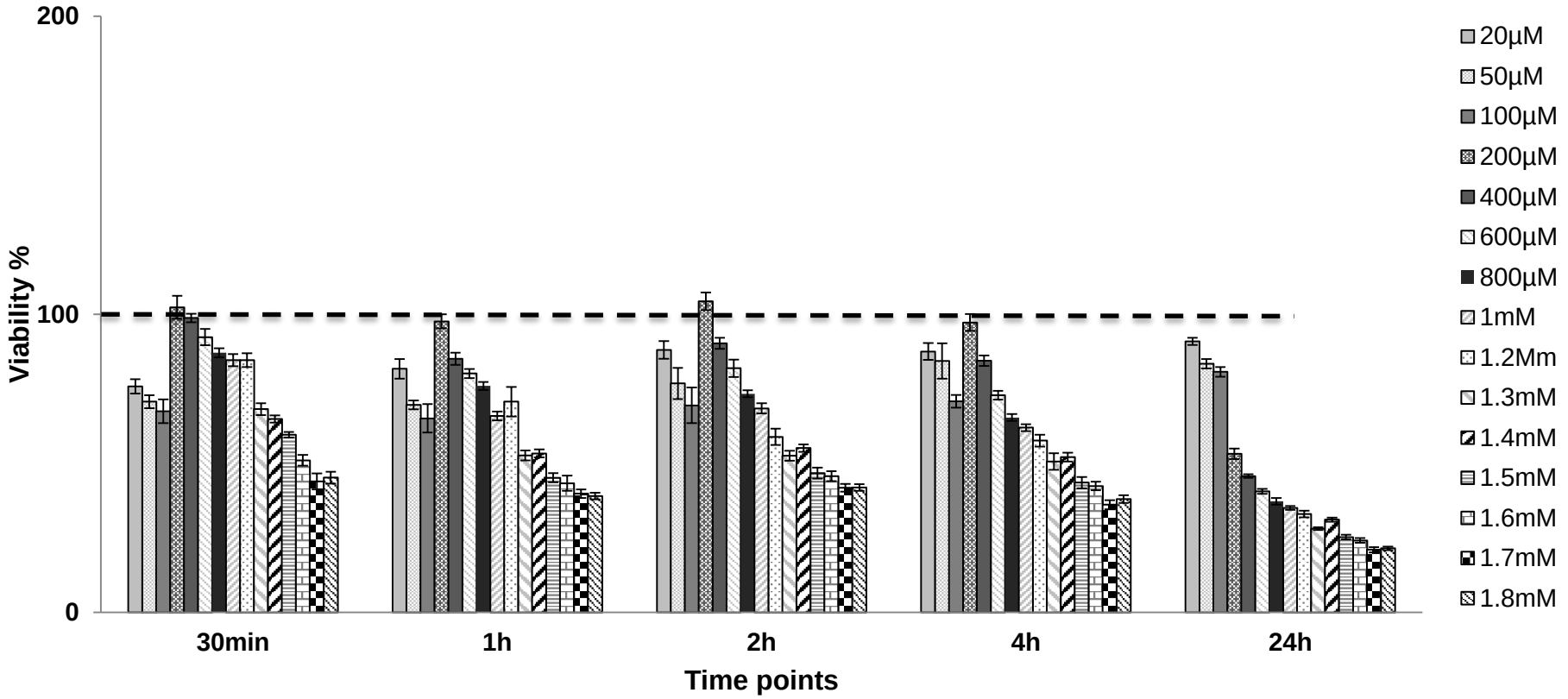

Figure B.9: Cellular viability of 3T3 cells exposed to different concentrations of amitriptyline (AMI), measured using alamarBlue®. Mean values ± SE (n=8) are shown for each experiment. Compared with untreated control cells (black dotted line).

Appendix C: SEM results of 3T3 cells were exposed to NMs

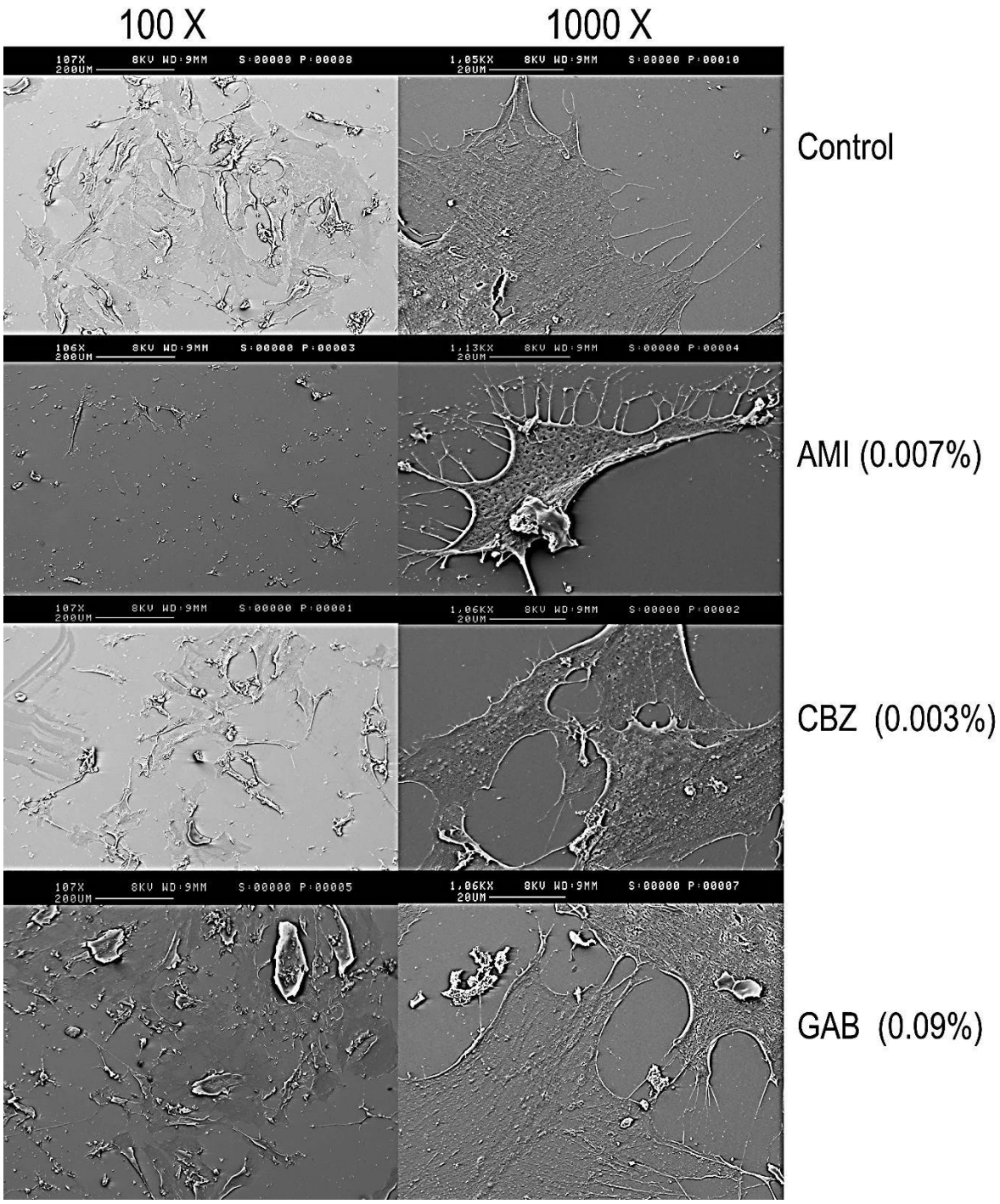

Figure C.1: SEM images at 100 X AND 1000 X magnification of 3T3 cells after 30 min exposure to amitriptyline (AMI), carbamazepine (CBZ) and gabapentin (GAB) at concentrations determined by HPLC.

Appendix D: Viability and cytotoxicity results of in-house constructed (Newcastle) 3D model

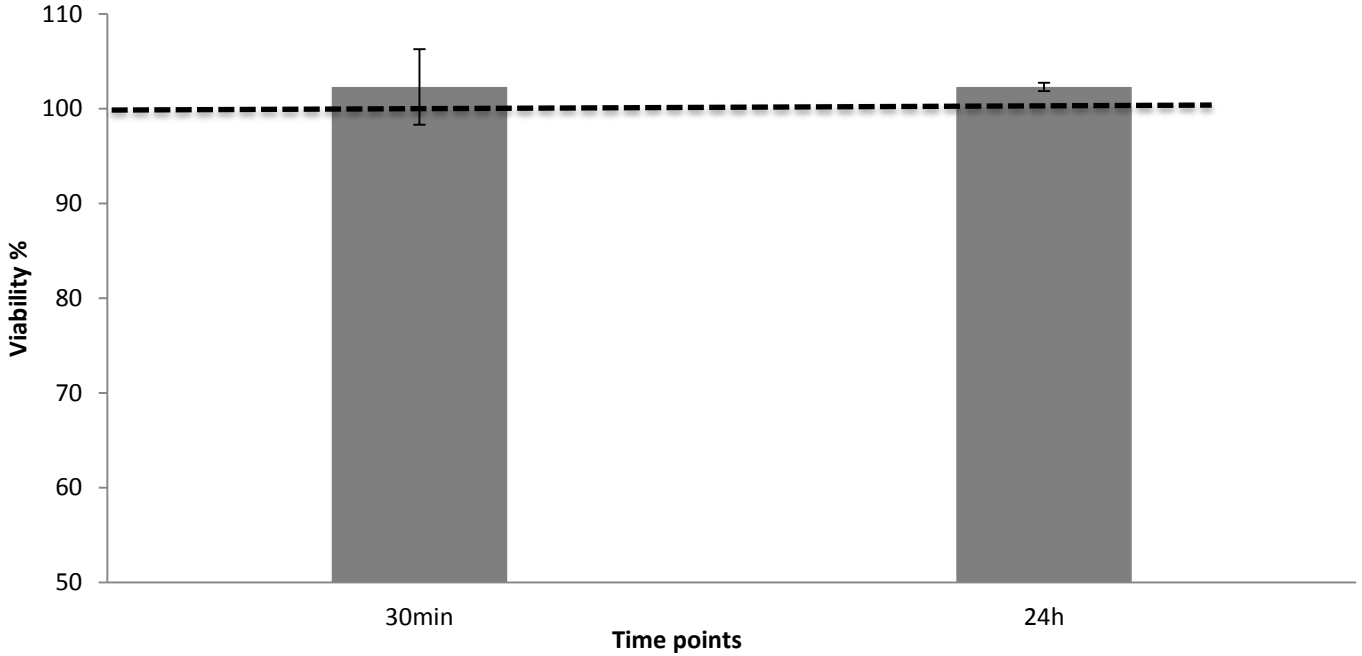

Figure D.1: Cellular viability of the in-house constructed 3D tissue (Newcastle models) was measured using alamarBlue®, exposed to amitriptyline (226 µM) at 30 min, 24 h time points. Mean values ± SE (n=3) are shown for each experiment compared with untreated control cells (black dotted line) by parametric independent t- test, *p>0.05.

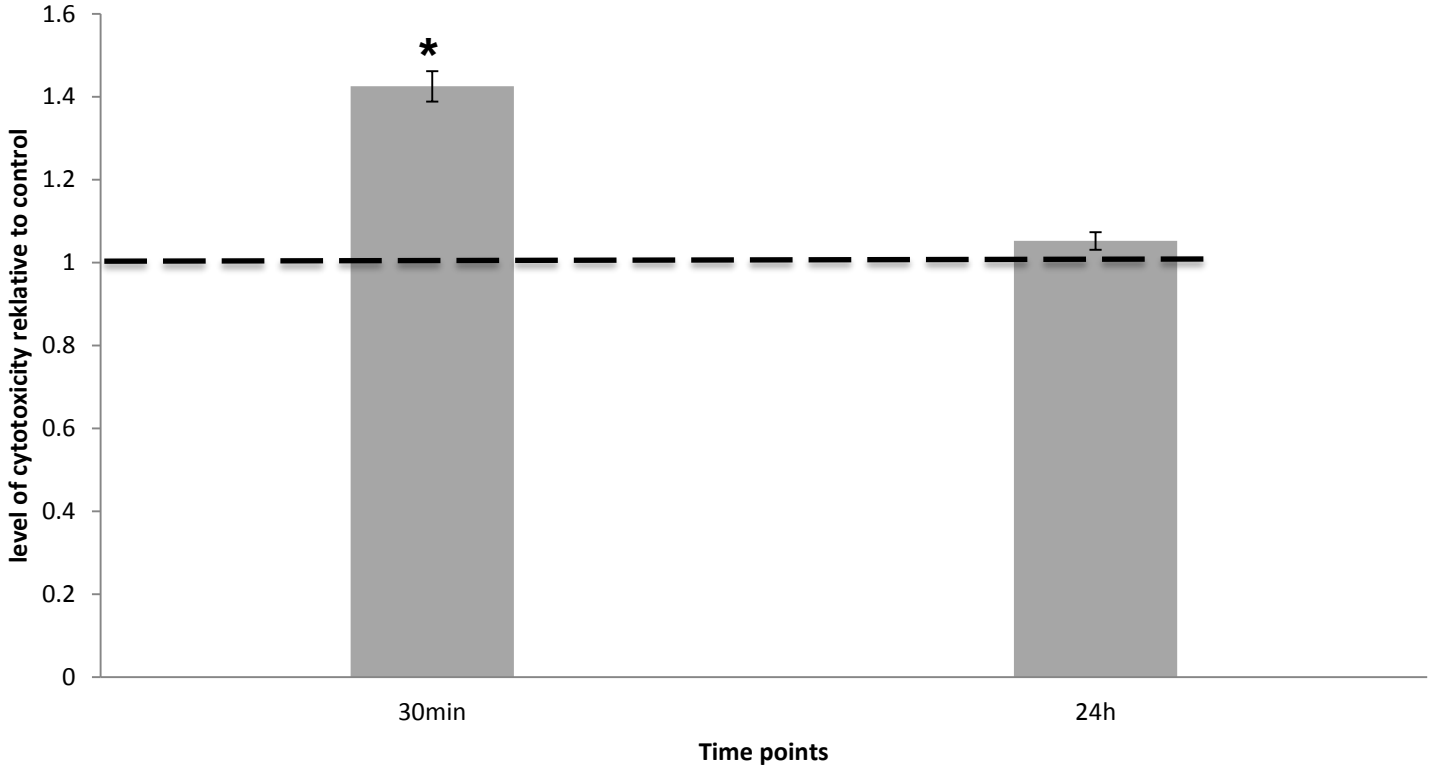

Figure D.2: Cytotoxicity results of the in-house constructed 3D tissue (Newcastle models) were exposed to AMI (amitriptyline), (226 µM) for 30 min, 24 h. Mean values ± SE (n=3) are shown for each experiment after were normalised to control results. Compared with control results at the same time points by non-parametric independent t-test, * $p<0.05$.

Appendix E : Publications and conference participations

1- Paper in press:

Al-Musawi, M., Durham, J., Whitworth, J.M., Stone, S.J., Nixdorf, D.R. and Valentine, R.A. (2016) 'Effect of topical neuromodulatory medications on oral and skin keratinocytes', J Oral Pathol Med.

2- Conference poster presentation/ ICOT 2013

Al-Musawi, M., Durham, J., Whitworth, J.M., Stone, S.J. and Valentine, R.A.: *In vitro* toxicity of Amitriptyline used as a topical agent in neuropathic orofacial pain.

3- Conference poster presentation/ IADR Boston 2015

Al-Musawi, M., Durham, J., Whitworth, J.M., Stone, S.J. and Valentine, R.A: In Vitro Effects of Topical Neuromodulatory Medication on Orofacial Tissue. Published abstract.

4- Conference poster presentation/ IADR Seoul 2016

Al-Musawi, M., Durham, J., Whitworth, J.M., Stone, S.J., Nixdorf, D.R. and Valentine, R.A.: Is Amitriptyline Safe as Topical Treatment for Neuropathic Orofacial Pain? In Press.

Prizes:

1- Poster prize at the quadrennial International Conference for Orofacial Pain and Temporomandibular disorders, ICOT/AAOP 2013.

2- 3rd Wiley-Blackwell Young Investigator Award in Neuroscience, IADR Boston 2015.

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