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An *ex-vivo* model to determine dental pulp responses to heat and light-curing of dental restorative materials

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ABSTRACT

Aim: Based on histological studies from the 1960s, it is recommended that dental pulp temperature increases should not exceed 5.5 °C. However, no contemporary reliable models exist to explore the effects of heat on living dental pulp. The aim of this project was to develop a clinically valid model for studying temperature increases caused by three commonly-used light curing units (LCUs).

Methods: Temperature increases caused by LCUs at varying exposure times and *via* various thicknesses of dentine were recorded using traditional approaches (*i.e.* thermocouple device on a laboratory bench) and an *ex-vivo* tooth slice model. Histomorphometric and immunohistochemical (IL-1β, HSP70, caspase-3) analysis was performed of the tooth slice model following varying exposure and culture times.

Results: Reduced dentine thickness and increased exposure time led to increases in temperature. Whilst the majority of temperature increases recorded using the traditional approach (53 of 60) were greater than the recommended 5.5 °C, 52 of the 60 reference points recorded using the *ex-vivo* tooth slice model resulted in temperature increases of less than 5.5 °C. Temperature increases of 5.5 °C or more that are prolonged for 40 s caused an immediate decrease in cell number. IL-1β was not detected in any samples, while HSP70 was detectable immediately after exposure to a temperature increase of 6 °C or more. Higher levels of HSP70 were detected after 24 h culture in tooth slices that experienced a temperature increase of 7.5 °C or more. Low levels of caspase-3 were detected in tooth slices exposed to temperature increase of 7.5 °C or more.

Conclusion: Experimental arrangements for assessing LCU performance that measure temperature increases using a thermocouple device on a laboratory bench should no longer be used. Future studies in this area should include replication of the clinical environment using greater sophistication, such as the use of an *ex-vivo* tooth slice model as described here. Temperature increases of 5.5 °C or more for 40 s caused an immediate decrease in cell number, which supports previous findings. However, complex interactions at an immunohistochemical level suggest that while temperature increases of 5 °C or less are ideal, there may be some cell damage between 5–7 °C which might not result in pulpal death. Further investigations are indicated.

1. Introduction

Despite recent advances in the prevention and management of dental caries, teeth with healthy pulps are often subjected to procedures which generate significant amounts of heat. An increasingly common source of potential thermal damage is the irradiation of remaining tooth tissues and underlying pulp with new high-powered light-curing units (LCUs) used for initiating polymerisation of resin-based composite (RBC) materials [1]. The use of RBCs has increased dramatically in general dental practice, and has been driven by the need for minimally invasive treatment of new lesions of caries and the replacement and repair of defective restorations [2–4]. Dental school teaching of RBC placement in posterior teeth has increased significantly in recent years [5], and RBCs are now recommended as the “material of choice” for restoring posterior teeth by dental teaching societies [6]. Further examples of dental procedures which involve heat generation include use of materials with exothermic setting reactions (*e.g.* provisional crown and bridge material) or frictional heat generated by cutting burs [7–9].

However, the biological effects of heat on the dental pulp and dentine are poorly understood. The single reference study in this area is

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that of Zach & Cohen which, in a histological study of dental pulps of teeth extracted from five subject Macau Rhesus monkeys, demonstrated that 15% of teeth heated by 5.5 °C, and 60% of teeth heated by 11.1 °C had histological features of irreversible pulpal damage [10]. However, while the threshold of a 5.5 °C temperature increase is regarded as being clinically significant, published data indicates temperature increases caused by newer LED LCUs exceed this threshold [11,12]. In clinical practice, many dentists no longer place insulating base cements under RBCs, possibly exposing the dentine-pulp complex to harmful irradiation from high-powered LCUs in deep cavities during restoration placement [13,14]. Studies demonstrate that significant heat generation in excess of 5.5 °C can also occur during cavity preparation using non water-cooled burs (11.64 °C) [7], newer laser-based tooth bleaching techniques (15.96 °C) [8], and setting reactions of exothermic materials, such as PMMA-based materials (39 °C) [9]. It is clear that either the method of determining the change (increase) in temperature, or the validity of the accepted 5.5 °C threshold is questionable. There is no contemporary reliable model to explore the effects of heat on living dental pulp.

The aim of this project is to develop a clinically valid model for studying temperature increases caused by three commonly-used LCUs. The effect of temperature increases on pulpal physiology will be studied within a 3D organotypic ex-vivo model. Comparison will also be made with existing, traditional models, of determining the clinical significance of heat increases from LCUs. It was hypothesised that temperature increases less than 5.5 degrees Celsius would not damage/disrupt normal pulpal physiology.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise stated, chemicals were obtained from Sigma (Dorset, UK). Dulbecco's Modified Eagle medium (DMEM), obtained from Invitrogen (Paisley, UK) was used for tooth slice culture. This was supplemented with 10% heat inactivated fetal calf serum (FCS), 0.15 mg/ml vitamin C, 200 mM L-glutamine (Invitrogen) and 1% antibiotics containing 1000 units/ml penicillin G sodium, 10 µg/ml streptomycin sulphate and 25 µg/ml amphotericin B (Invitrogen).

2.1.1. Temperature increases measured using traditional models

Three light curing units (LCUs) were used (two light emitting diodes (LED) LCUs and one Quartz Tungsten-halogen (QTH)). The power output and commercial details of each of LCU are listed in Table 1.

A digital radiometer was used to check the power output of the LCUs during experiments. The tip of a micro-thermocouple (a basic type K thermometer (sper scientific 800011)), was placed directly below the LCU to measure temperature change. The temperature changes of each LCU were recorded at increasing time intervals of 10 s, 20 s, 30 s and 40 s, with varying thicknesses of dentine between the LCU and thermocouple (Table 2). The thermocouple was allowed to cool down to room temperature between each measurement.

Dentine slices were introduced between the LCU and the thermocouple. Dentine slices were obtained from incisor teeth extracted from 28 day old male Wistar rats. Incisors were washed in sterile culture media and 2 mm long sections prepared using the lingual side of the upper incisors only, using a cooled low speed diamond saw (TAAB® Laboratories equipment Ltd, Brekshire, UK). The thickness of the dentine slices were measured using the Digital Caliper, 300 mm, DURATOOL®. Varying thicknesses were introduced (0.4 mm, 0.79 mm, 1.39 mm, 1.62 mm). The tooth slices were placed directly above the thermocouple. The temperature changes of each LCU were recorded at increasing time intervals of 10 s, 20 s, 30 s and 40 s. The results are reported in Table 2.

Table 1
Power output and commercial details of Light Curing Units (LCUs) used in this study.

Light curing unit	Type of light curing unit	Power (mW/cm^2)	Manufacturer
Coltolux 75	Quartz Halogen	1380	Coltene Whaledent
Coltolux	Light emitting diodes	1039	Coltene Whaledent
Ultra Lume	Light emitting diodes	800	Ultradent

2.1.2. Temperature increases measured using novel ex-vivo models

In this experiment the conditions in 'traditional' approaches described above were reproduced in an *ex-vivo* tooth slice model.

2.2. Preparation of tooth slices

Upper and lower incisors were dissected from 28 day old Male Wistar rats sacrificed by Schedule 1 procedure as previously described [15,16]. Incisors were cut into 2 mm thick transverse sections using a diamond-edged rotary bone saw (TAAB, Berkshire, UK), embedded in 1% low melting point agar prepared using supplemented DMEM and transferred to Trowel-type culture. Tooth slices were cultured in supplemented DMEM at 37 °C, 5% CO2 for 24 h to prevent any initial cellular response to the cutting process from interfering with experimental observations.

2.3. Exposure of tooth slices to light curing units

Following 24 h culture, tooth slices were exposed to the LCUs (Table 1). A thermocouple was inserted into the tooth slice to measure the temperature change after 10, 20, 30 and 40 s exposure. The experiment was repeated with varying thickness of dentine between the LCU and tooth slice (Table 3).The same thicknesses of dentine as used as in the traditional experiment.

2.3.1. The effect of temperature change caused by dental light-curing units on pulpal cells in an ex-vivo tooth slice model

Tooth slices were prepared as previously described [15,16]. Following 24 h culture, tooth slices were exposed to LCUs for up to 60 s. In addition, to increase clinical relevance, the effect of dental adhesives and composites were also considered. Tooth slices were exposed directly to the LCUs, or coated with Prime&Bond®NT (Dentsply, Surrey, UK), or covered with 0.5 mm devitalised dentine coated with Prime&Bond®NT and 1 mm A2 Grandio restorative material (VOCO, Cuxhaven, Germany). To prevent disturbance of the normal tissue architecture, a thermocouple was not inserted into the tooth slice.

The slices were processed according to the following protocols:
Immediate fixation following:

- direct exposure for 20 or 40 s

24 h culture following:

- direct exposure for 20, 40 or 60 s
- 20 s with Prime & Bond
- 20 s + 0.5 mm dentine slab, Prime & Bond + 1 mm thickness composite

2.4. Histological analysis of tooth slices

Following culture the tooth slices were processed for histological analysis, as described elsewhere [16]. Tooth slices were fixed in 10% (w/v) neutral buffered formalin, demineralized in 10% (w/v) formic acid and dehydrated through a series of alcohols, prior to embedding in paraffin wax. 5µM sections were cut and stained with hematoxylin and

Table 2
Mean temperature change recorded with various thicknesses of dentine slices (at room temperature) between the LCU and thermocouple (n = 10 for each time and LCU). Temperature changes greater than the recommended 5.5 °C are shaded (=53 of 60 data points).

LCU	Thickness of dentine (mm)	Mean temperature change (°C)			
		10 seconds	20 seconds	30 seconds	40 seconds
Coltolux 75	0	15.7 (0.916)	16.4 (1.065)	17.2 (0.781)	17.7 (0.816)
	0.4	14.8 (0.782)	15.7 (0.923)	16.5 (0.85)	17 (0.632)
	0.79	13.9 (0.743)	14.8 (0.46)	15.5 (0.5)	16.05 (0.471)
	1.39	13.4 (0.489)	14.4 (0.537)	15.2 (0.502)	15.9 (0.489)
	1.62	13.3 (0.46)	14 (0.447)	14.9 (0.489)	15.7 (0.509)
Coltolux LED	0	8.5 (0.35)	9.6 (0.35)	10.4 (0.3)	11.05 (0.35)
	0.4	6.4 (0.502)	7.5 (0.632)	8.3 (0.509)	9.1 (0.583)
	0.79	5.6 (0.374)	6.7 (0.39)	7.6 (0.522)	8.3 (0.458)
	1.39	5.5 (0.316)	6.4 (0.3)	7.2 (0.458)	8 (0.387)
	1.62	4.9 (0.489)	5.8 (0.458)	6.8 (0.335)	7.5 (0.387)
Ultra Lume LED	0	6.6 (0.489)	7.6 (0.374)	8.4 (0.374)	9.2 (0.39)
	0.4	5.5 (0.61)	6.4 (0.663)	7.2 (0.64)	7.9 (0.55)
	0.79	5.2 (0.812)	6.3 (0.509)	7.1 (0.538)	7.9 (0.624)
	1.39	4.4 (0.776)	5.3 (0.844)	6.1 (0.916)	6.9 (0.834)
	1.62	4.4 (0.374)	5.6 (0.374)	6.7 (0.39)	7.6 (0.415)

eosin (H&E), and viewed under a light microscope, with images captured using a Nikon digital camera and ACT-1 imaging software. For each experimental condition, 3 tooth slices were sectioned and imaged. Five random fields of view (RFV) were taken within each section and the nuclei counted in five 50 μm2 areas in each RFV to obtain an average nuclei number for each experimental condition. All software settings remained the same for each tissue section and culture, and initial measurements when calibrating the software were validated by manual counts and proved consistent with manual assessment. Mean values were analyzed using one-way analysis of variance and Tukey's post hoc test to analyze differences in cell number.

2.5. Immunohistochemical analysis of light cured tooth slices

Tooth slices were processed and sectioned as previously described, mounted on Super-Frost microscope slides (Fisher Scientific, Loughborough, UK) and dried overnight at 65 °C. Sections were de-paraffinized with xylene for 10 min, washed with industrial methylated spirit (IMS) for 5 min, and re-hydrated in tap water for 5 min. Antigen retrieval was achieved by incubation with 25 μg/ml Proteinase K for 10 min at 37 °C. Non-specific binding was blocked with 1% bovine serum albumin (BSA) in tris-buffered saline (TBS) for 1 h. Following 3 x 2.5 min washes in TBS, sections were incubated with one of the following primary antibodies diluted appropriately in TBS for 18 h at 4 °C: mouse monoclonal anti-human heat-shock protein 70 (HSP70; clone 3A3, 1:50, Santa Cruz Biotech), rabbit polyclonal anti-human cleaved caspase-3 (clone ASP175, 1:1000, New England BioLabs) or goat polyclonal anti-rat interlukin-1β (IL-1β; clone R-20, 1:50, Santa Cruz). Sections were washed in TBS (3 x 2.5 min) prior to incubation in the dark for 1 h with the appropriate FITC conjugated secondary antibody diluted in TBS (1:100; Santa Cruz) and a bisbenzimide nuclear counterstain. Sections were dehydrated in IMS for 5 min and cleared in xylene for 5 min prior to mounting with fluorescent mounting medium (Dako, Stockport, UK). Sections were viewed by fluorescence microscopy using a Nikon digital camera as previously described. For negative controls, primary antibodies were excluded or replaced with an IgG isotype control diluted to the working concentration of the primary antibody.

3. Results

3.1. Measuring temperature increases using traditional approaches

The measurement of temperature increases with no intervening material, and with intervening dentine thicknesses between the LCU and micro-thermocouple, are reported in Table 2. Very large temperature increases were noted across all LCUs and exposure times (as great as 17.7 °C for the Coltolux 75 unit after 40 s) when no dentine slabs were included. Increasing the thickness of dentine led to decreased temperature increases, while increased exposure time led to increased changes of temperature. Without any dentine thicknesses, all of the temperature increases were greater than the recommended 5.5 °C arising from the work of Zach & Cohen. When intervening dentine thicknesses were considered, all of the temperature increases for the Coltolux 75 LCU, and most of the increases for the Coltolux LED and UltraLume LED were greater than the recommended 5.5 °C threshold.

Table 3
Mean temperature change recorded with various thicknesses of dentine slices (at room temperature) between the LCU and thermocouple (n = 10 for each time and LCU) in an ex-vivo tooth slice model. Temperature changes greater than the recommended 5.5 °C are shaded (=8 of 60 data points).

LCU	Thickness of dentine(mm)	Mean temperature change (°C)			
		10 seconds	20 seconds	30 seconds	40 seconds
Coltolux 75	0	5.9 (0.603)	7.4 (0.513)	8.05(0.568)	8.5 (0.611)
	0.4	4.1 (0.374)	5.3 (0.928)	6.7 (0.807)	7.6 (0.65)
	0.79	3.6 (0.471)	4.6 (0.471)	5.3 (0.556)	6.05 (0.471)
	1.39	2.6 (0.567)	3.6 (0.567)	4.1 (0.624)	4.9 (0.45)
	1.62	3.2 (0.754)	2.7 (0.754)	3.8 (0.565)	4.3 (0.612)
Coltolux LED	0	3.8 (0.568)	4.8 (0.537)	5.5 (0.643)	6.3 (0.636)
	0.4	3.2 (0.483)	4.3 (0.589)	4.9 (0.459)	5.4 (0.474)
	0.79	2.6 (0.437)	3.5 (0.408)	4.2 (0.483)	4.8 (0.54)
	1.39	1.3 (0.421)	2.4 (0.411)	3 (0.408)	3.7 (0.241)
	1.62	1.4 (0.337)	1.7 (0.258)	2.2 (0.474)	3 (0.471)
Ultra Lume LED	0	3.2(0.421)	4.05 (0.437)	4.7 (0.474)	5.5 (0.527)
	0.4	2.7 (0.411)	3.6 (0.316)	4.3 (0.349)	5.05 (0.376)
	0.79	2.8 (0.483)	3.9 (0.337)	4.7 (0.383)	5.5 (0.497)
	1.39	3.2(0.625)	4 (0.666)	4.7 (0.529)	5.5 (0.577)
	1.62	2.4 (0.529)	3.3 (0.485)	4.05 (0.643)	4.8 (0.54)

Within Table 2, 53 of the 60 reference points featured temperature increases greater than the recommended 5.5 °C.

3.2. Measuring temperature increases using the ex-vivo tooth slice model

The measurement of temperature increases within the novel ex-vivo tooth-slice model are reported in Table 3. A large reduction in the overall sizes of the temperature increases were noted in this experiment compared to the traditional arrangements. Within this model, 8 of the 60 reference points exceeded the recommended 5.5 °C, which mainly related to the more high-powered Coltolux LCU, at reduced distances and increased exposure times.

3.3. Histomorphometric and immunohistochemical analysis

3.3.1. Cell counts

Histomorphometric analysis of tooth slices exposed to LCUs showed an immediate decrease in cell numbers which was dependent on exposure time. Tooth slices which were returned to culture for 24 h following exposure to LCUs showed further decreases in cell number. Tooth slices treated with DBA alone, or DBA in combination with dentine slabs and RC were more resistant to cell damage. Fig. 1 shows cell counts following direct exposure for 20 or 40 s followed by immediate fixation. Fig. 2 shows cell counts following direct exposure for 20, 40 or 60 s, or exposure for 20 s *via* a layer of 'Prime & Bond NT' or a compound layer of dentine, 'Prime & Bond NT', and composite. After this exposure the tooth slices were cultured for 24 h and then fixed. Temperature increases of 5.5 °C or more that are prolonged for 40 s caused an immediate decrease in cell number. Some histological images of interest are shown in Figs. 3–5.

3.3.2. Immunohistochemistry

IL-1β was not detected in any samples.

HSP70 was detectable immediately after exposure in tooth slices that experienced a temperature increase of 6 °C or more. Higher levels of HSP70 were detected after 24 h culture in tooth slices that experienced a temperature increase of 7.5 °C or more (Fig. 6).

Low levels of caspase-3 were detected in tooth slices exposed to the Coltolux 75 LCU which experienced temperature increase of 7.5 °C or more (which was for 20, 40 and 60 s, noted on both immediate fixation and following fixation following 24 h culture after exposure) (Fig. 7).

4. Discussion

The results of this study are of relevance to clinicians, researchers and industry leaders. The use of resin-based composites, particularly for restoration of often extensively damaged posterior teeth, is continually increasing [3]. In particular, the implementation of the recommendations of the Minimata Treaty, let alone an increasing desire to practice minimally invasive operative dentistry *via* the selection of resin composite in preference to amalgam, would drive an increased use of exothermic light curing units in coming years [17–20].

At the outset, this study has considered the appropriateness of testing models for temperatures increases from LCUs and other heat

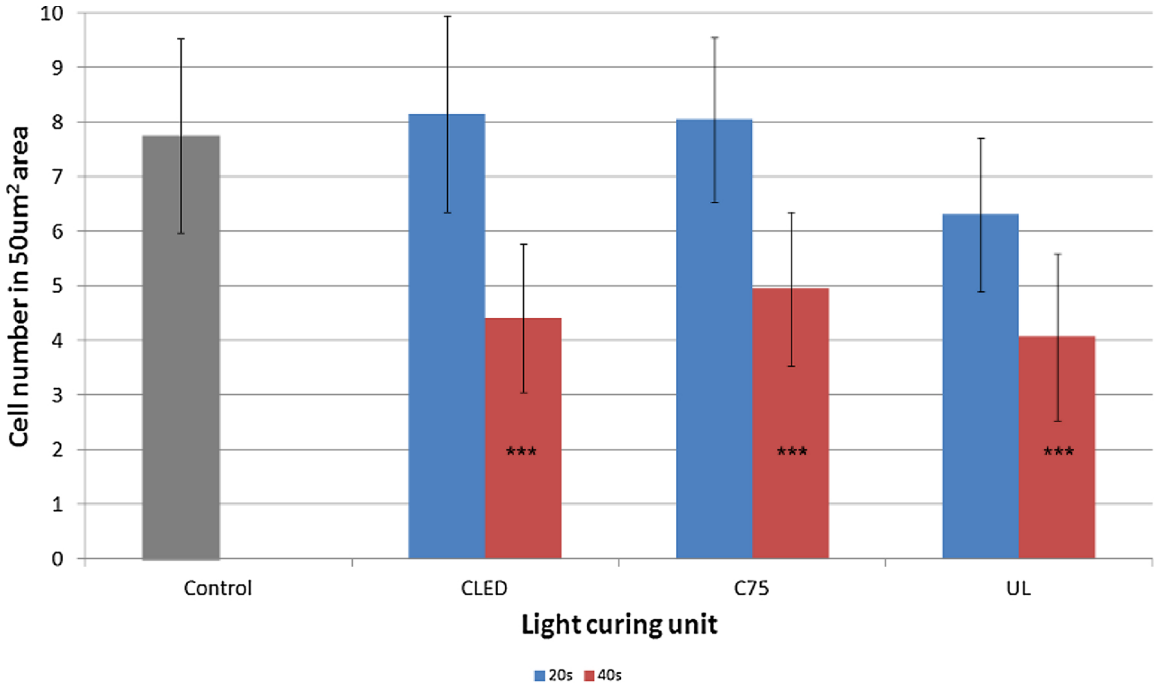

Fig. 1. Cell counts following direct exposure for 20 or 40 s followed by immediate fixation (error bars = standard deviations; *** = $p < 0.001$).

sources, and answers the vexed question as to why, in the knowledge of the 5.5 °C limit established by Zach & Cohen [10], the apparent significant temperature increases - often greater than 10 °C - do not cause significant pulpal damage and death in clinical practice. Comparison of various experimental arrangements has demonstrated that assessments of temperature increases involving a thermocouple on a laboratory bench are of little value to understanding the clinical performance of a new light curing unit or other source of heat, such as burs or exothermic setting reactions. Within this study, the significant temperatures increases seen when the LCUs were assessed using traditional *in vitro* arrangements were not seen with the same LCUs and protocols were transferred into the *ex-vivo* model. It is therefore recommended that *in vitro* testing arrangements are of little value to clinical understanding and should no longer be used. We would recommend that future studies which measure temperature increases should include replication of the clinical environment using greater sophistication: the use of an *ex-vivo* tooth slice model, as described here, would seem a natural way forward for this.

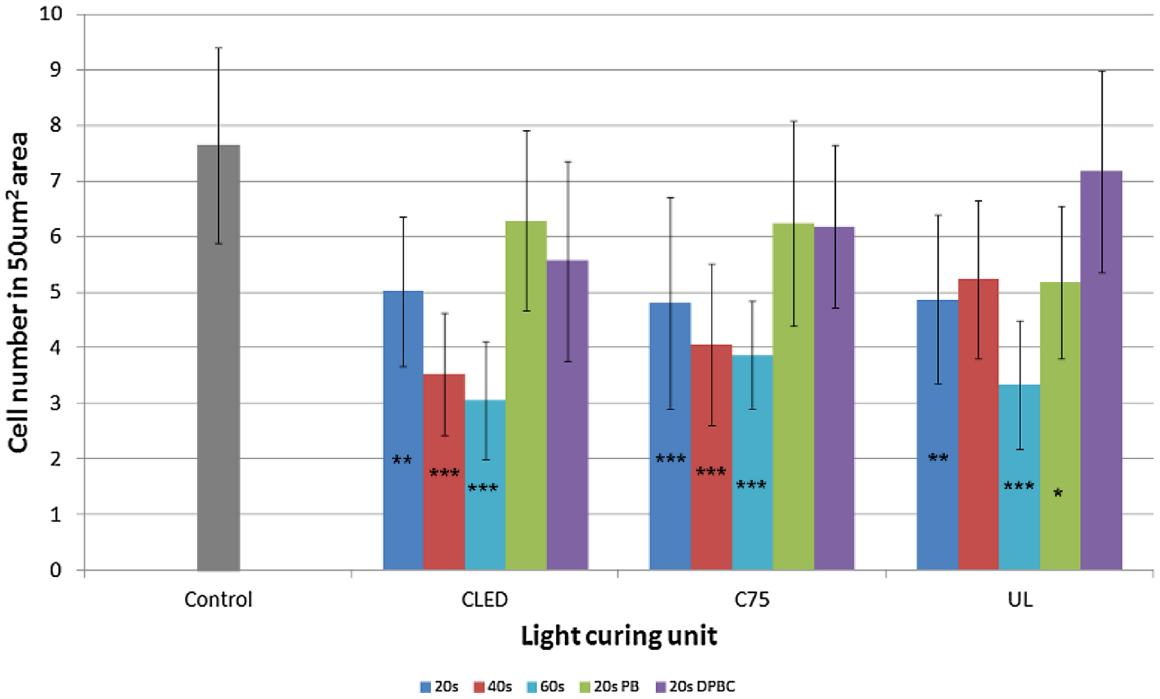

Fig. 2. Cell counts following direct exposure for 20, 40 or 60 s, or exposure for 20 s *via* a layer of 'Prime & Bond NT' or a compound layer of dentine, 'Prime & Bond NT', and composite. After this exposure the tooth slices were cultured for 24 h and then fixed (error bars = standard deviations; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

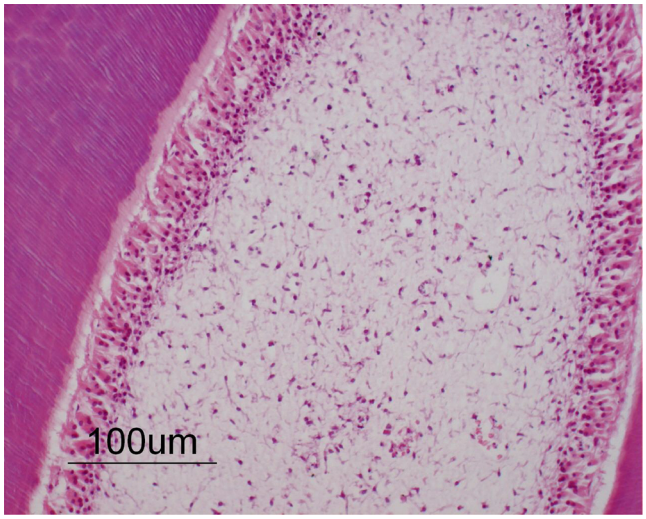

Fig. 3. Example of control specimen (no LCU irradiation). This shows normal pulpal and odontoblast architecture.

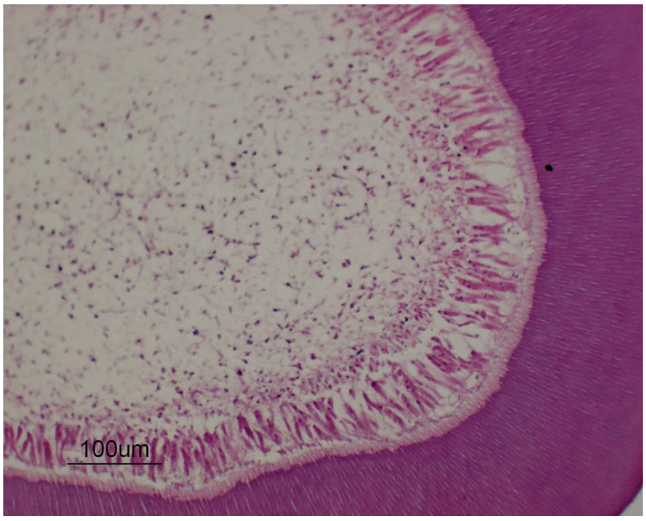

Fig. 4. Section showing pulp following irradiation using the Coltolux LED for 30 s. Odontoblast damage is visible in the upper part of this section (x20 magnification). From Experiment #3, the temperature change in this setting was 5.5 °C.

The *ex-vivo* tooth slide model offers many advantages in understanding the performance of heat producing sources, not least that the method is validated and reduces the unnecessary sacrifice of a large number of animal subjects. The reference study of Zach & Cohen, 10 involved application of a heat source (a soldering iron) to the labial surfaces of tested teeth within 5 Macau Rhesus monkeys. While the study was novel for its era, the outcome measurement was the amount of histological evidence of pulpal damage (15% of teeth heated by 5.5 °C, and 60% of teeth heated by 11.1 °C had histological features of irreversible pulpal damage, hence the origin of the 5.5 °C threshold). Following application of heat the teeth were extracted prior to sectioning of teeth and histopathological examination. In contrast, the *ex-vivo* tooth slice model avoids the potential confounding effects of animal sacrifice, tooth extraction and the trauma of sectioning during sample preparation, on the viability and health of the pulpal tissues, allowing a more relevant understanding of the physiological and pathological effects of heat on the dentine-pulp system.

In this study, temperature increases of 5.5 °C or more that are prolonged for 40 s caused an immediate decrease in cell number, which supports previous findings. Expression of HSP70 (heat shock protein

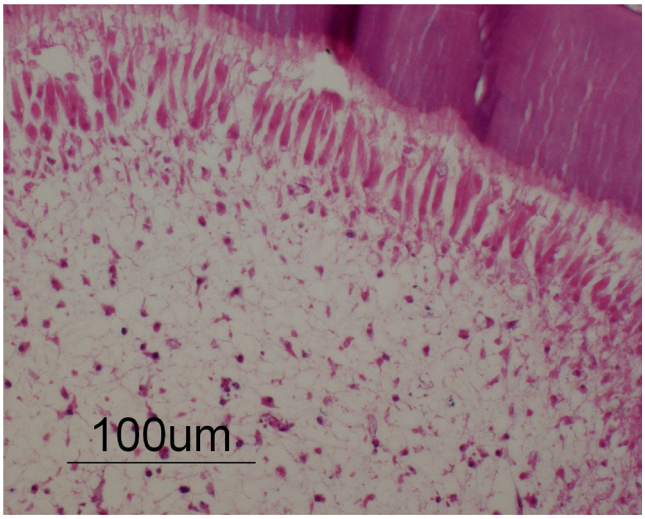

Fig. 5. Section showing pulp following irradiation using the Coltolux 75 LCU for 30 s. Odontoblast damage is visible in the upper part of this section (x 40 magnification). From Experiment #3, the temperature change in this setting was 8.05 °C.

70) was observed as a response to damage when temperatures inside the pulp exceed 6 °C. Heat shock proteins are evolutionarily conserved proteins that can be induced by stress signals, including environmental stresses and pathophysiological states (*e.g.* inflammation and infection) [21]. They function as chaperones assisting with protein folding to protect cells from protein denaturation or cell death under stress conditions. As such they stabilize intracellular structure and are critical for cell survival, playing a role in the anti-apoptotic process, its promotion as well as regulation of important pro-inflammatory transcription factors [22–24]. They are termed according to their molecular weight, and HSP70 functions to maintain tissue homeostasis and is physiologically expressed during tissue trauma. In the tooth, HSP70 has been reported to be expressed during reparative dentin formation [25] and in the dental pulp following trauma [26,27]. Our observation of expression of HSP70 within pulp tissue where temperature exceeded 6 °C concurs with previous findings and suggests the presence of cells that are not dead or undergoing apoptosis, but have been stressed through exposure to increased temperature. Expression of HSP70 is maintained for 24 h in pulps where the temperature is increased by 7.5 °C or more. Okai et al. [28] demonstrated that pulpal fibroblasts exposed to heat stress markedly upregulated HSP70 and in conjunction with our findings, suggests heat stress may increase the wound healing capacity of dental pulp cells.

When temperature increases exceeded 7.5 °C, which occurred only with the C75 unit, a low level of caspase-3 was also detected. This indicates that whilst HSP70 may infer some protection from apoptosis, there is an upper limit at which this will occur. Caspase-3 is activated in the apoptotic cell, and has been suggested to be a key role in a cell's apoptotic machinery [29]. Apoptosis may occur through two main specific signalling pathways, the extrinsic and intrinsic pathway. The extrinsic pathway is induced by an external signal stimulated by receptors (*e.g.* death receptors) and is triggered through activation of caspase 8 (initiator) and caspase 3 (effector). The intrinsic pathway is mediated by mitochondria and release of pro-apoptotic that activate caspase 9 signalling that triggers caspase 3 activation. In our study, low levels of caspase-3 suggest minimal cell apoptosis and concurs with the expression of HSP70.

Due to the complex cascade of events that are involved in the inflammatory response, there may be a relationship between HSP-70 and IL-1β release. HSPs have positive and negative effects in regulating macrophage function and extracellular HSPs may stimulate the immune response, whereas intracellular HSPs could function as a negative

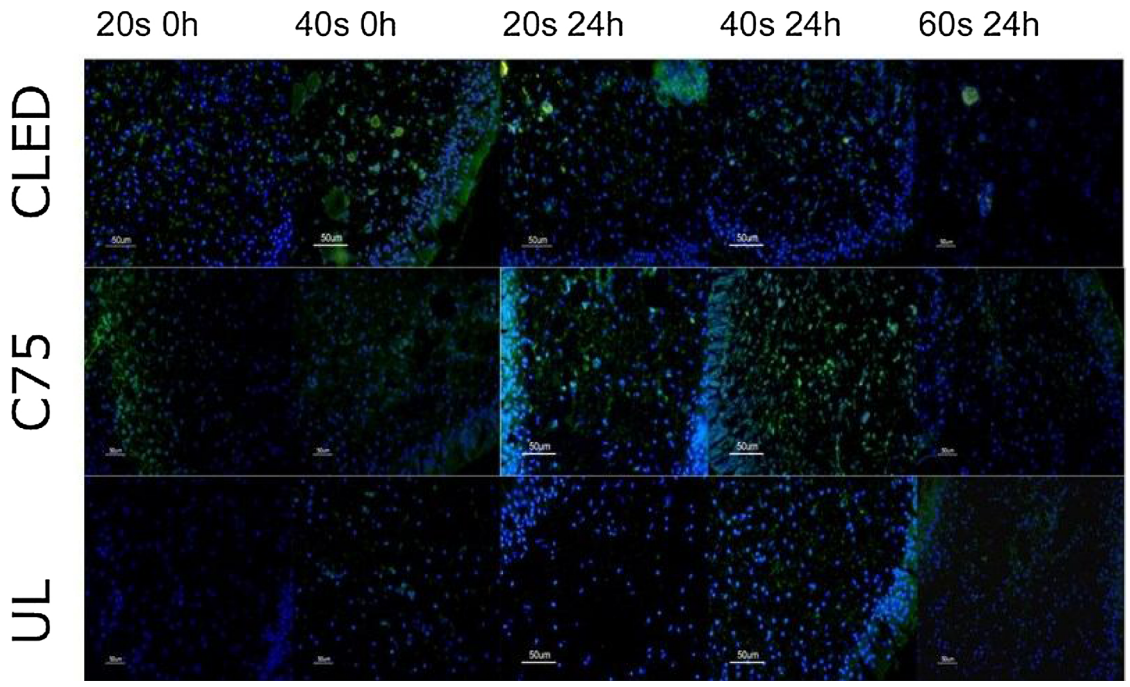

Fig. 6. HSP70 was detectable immediately after exposure in tooth slices that experienced a temperature increase of 6 °C or more. Higher levels of HSP70 were detected after 24 h culture in tooth slices that experienced a temperature increase of 7.5 °C or more (HSP70 stains green in the image, blue represents the nuclear counterstain). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

regulator to control the inflammation [30]. Extracellular HSPs can also stimulate the release of TNF-α, and IL-1β, amongst other cytokines by monocytes/macrophages [31]. Our data suggests HSP-70 provides further protection *via* possibility limiting the development of pulpal inflammation at lower temperatures but above 5 °C. Thermally induced HSP70 may regulate cytokine secretion (including IL-1 β) and it is possible that the effect of heat is determined by the specific activation state of macrophages in the tissue [32]. While we were unable to detect IL-1 β in our samples, this may be due to levels being below those detectable or the lack/low numbers of macrophages in the *ex vivo* tissue slice.

It is worth noting that the addition of DBA or curing through dentine and RC appeared to reduce the thermal damage to the tooth slice. This is likely to be a result of the increased distance between the LCU and the pulp and the insulating effects of the RC and DBA. The findings of this study are of relevance given the increased trend towards the clinical placement of resin based composites [33,34].

5. Conclusions

Based on the findings of this study, experimental set-ups that measure temperature increases using a thermocouple device on a laboratory bench, without the use of an ex-vivo tooth slice model, are of little value to clinical understanding and should no longer be used. We would recommend that future studies which measure temperature increases should include replication of the clinical environment using greater sophistication: the use of an *ex-vivo* tooth slice model, as described here, would seem a natural way forward for this as it provides a 3-dimensional environment of multiple pulpal cell types and tissue matrix.

Temperature increases of 5.5 °C or more that are prolonged for 40 s caused an immediate decrease in cell number, which supports previous findings. However complex interactions are suggested around the levels of release of Heat Shock Proteins and Cleaved Capaspe-3. The levels of HSP seen would suggest numerous cells of the dental pulp become stressed/damaged when temperatures exceed 5.5C, but these cells are not undergoing active apoptosis or are dead. It is possible that HSP70 may also confer protection against apoptosis by limiting cleaved capaspe-3. However, when temperatures reach 7.5 °C such protection is lost. Therefore these results suggest a possibility that while temperature increases of 5 °C or less are ideal, there may be some cell damage between 5–7 °C which may not result in pulpal death but lead to thermal

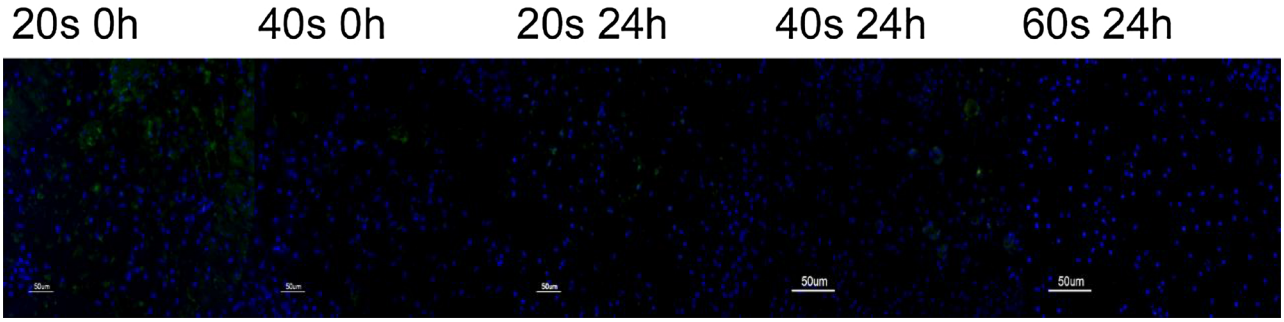

Fig. 7. Low levels of caspase-3 were detected in tooth slices exposed to the Coltolux 75 LCU which experienced temperature increase of 7.5 °C or more (which was for 20, 40 and 60 s, noted on both immediate fixation and following fixation following 24 h culture after exposure). (Caspase-3 stains green in the image, blue represents the nuclear counterstain). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induced stress. Further investigations over longer time periods and a great range of temperatures are indicated.

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