Thermal damage behaviour of human dental pulp stem cells

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Objective

This study was designed to examine the influence of temperatures ranging from 37 to 65 °C on the cell morphology of DPSC stem cells via light and electron microscopy, a synthesis of Heat Shock Proteins (HSP) with fluorescence-marked antibodies and vitality via the Life/Dead Fluorescence Kit.

Table 1: Life/Dead Assay 1 hour und 24 hours after thermal treatment.

Temperature	Letality %	
°C	1 h	24 h
37	0	0
39	0	0
42	0	0
45	0	0
46	0,5	2
47	10	17
48.5	18	29
50	17	27
55	24	59
56.5	48	54
58	100	100
60	100	100
65	100	100

Table 1

Material and methods

DPCS were cultivated at 37 °C and 5 % CO_2 in sterile cell chambers (MiniCeM, JenLab GmbH, Jena, Germany). The cells were irrigated with pre-heated culture medium (Eagle's MEM, Gibco BRL, Paisley, Scotland, 37 °C) with 20 % FCS, 2 mM L-Glutamin and 100 µM L-Ascorbate-2-Phosphate in order to remove cellular debris previously to the temperature trials. Filling the chamber with the culture medium followed and a preheated water bath of different temperatures was introduced. Up to an incubation temperature of 46 °C, the experiments were conducted with temperatures rising every 2 °C and 0.5 °C in the sensitive temperature scale of 46 °C to 58 °C. In addition, trial series were carried out at 60 °C

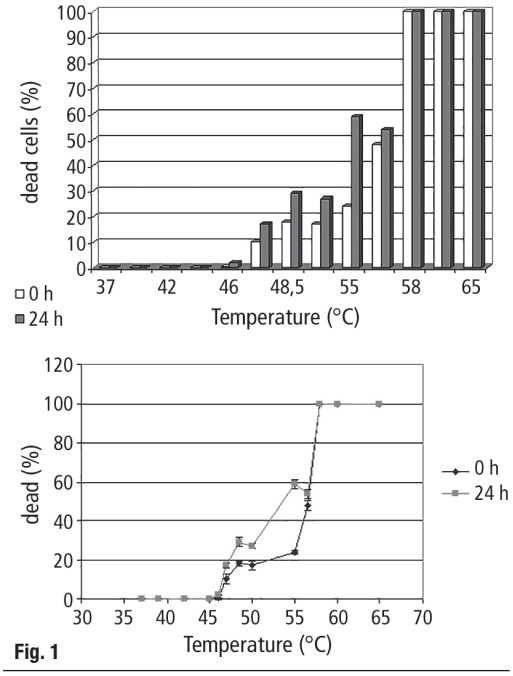


Fig. 1: Vitality test of thermally treated DPSC.

Fig. 1

and 65 °C. After a total of 15 min of thermal treatment, the cells were cooled down in the incubator at a temperature of 37 °C for 1 hour.

Some of the cells which had undergone thermal treatment were examined with the Life/Dead Fluorescenc Assay (Molecular Probes, Eugene, OR, USA) in order to assess vitality via fluorescence microscopy and Axiovert 200 (ZEISS, Jena) after incubation. A mixture of 2 µM Calcein AM and 4 µM Ethidium-homodimer-D1 was added to the cells which were slowly cooling down at 37°C in the incubator either 1 h or 24 h after thermal treatment and incubated for 10'. Vital cells exhibited a green fluorescence caused by calcein, while lethal cells showed a red core fluorescence (Ethidium-homodimer-D1 and coupled DNA). 100 cells of each type were enumerated.

In order to examine the synthesis of HSP proteins, the cells having undergone thermal treatment were processed as follows:

- Opening of the chamber and removal of the coverslip containing the cells
- Suction of the nutritive medium, two rinses with PBS (isotonic: 67 mM phosphate buffer pH 7.2–7.4, 0.5 % NaCl)
- 12' fixation in 2 % paraformaldehyde in 0.1 M cacodylate buffer pH 7.2; Rinse: 3 x PBS, 2 x TBS (Tris buffered saline, 50 mM Tris-HCl buffer, 1.25 % NaCl)
- Parting of the coverslip with Pap-Pen pen (oil pen), possibly correct with paraffin
- Incubate one half of the coverslip overnight at 4 °C with 1:500 diluted antibody AK HSP25, Rabbit (Biomol), diluting solution: fish gelatin 1 %, Triton x 100 1 % in TBS)
- Cover the other half of the coverslip exclusively in diluting solution (without AK)
- Wash in TBS for 3 x 10'
- Conjugate with the second antibody AK Anti-Rabbit-Alkaline Phosphatase for two hours at room temperature (Ziege, dilution: 1:50 with fish gelatine 1 % and Triton X 100 1% in TBS)
- Wash in TBS for 3x10'
- 15` Alkaline-Phosphatase verification with 3 mM Levamisol in Chedium (induces blue-brown colouring according to Seidel).

In order to perform examinations with scanning electron microscope, the cells were processed as follows:

- Washing of the cells in cacodylate buffer (0.1 M)
- Fixation with 2.5 % Glutaraldehyde in cacodylate buffer for 20'
- Washing with cacodylate buffer for two times, followed by two washings with Aqua dest
- Dehydration with increasing alcohol concentration: 20 %, 30 %, 50 %, 70 %, 90 %, 2x in 100 % EtOH for 10' each

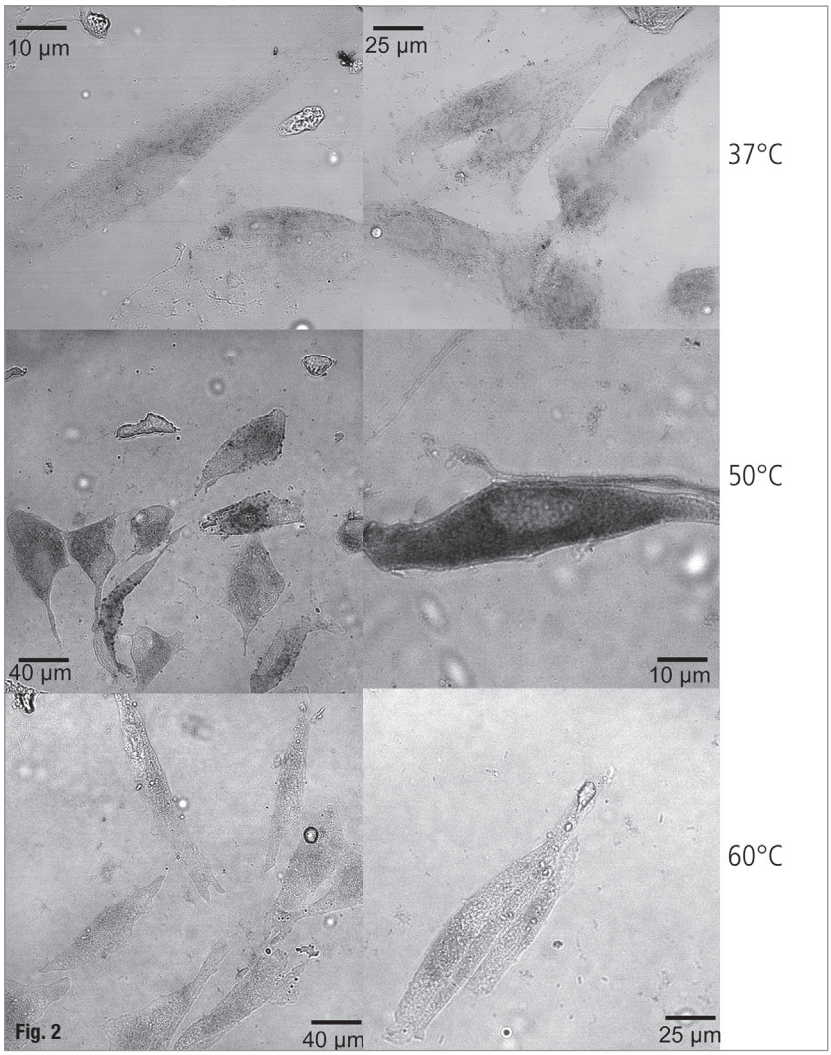


Fig. 2: HSP-detection caused by an antibody color reaction.

- Further processing of the samples at the Centre for Electron Microscopy (Critical Point Drying and sputtering with gold; SCD 005, BAL TEC AG, Liechtenstein)
- Microscope: Zeiss EM 902 A.

Examinations with the transmission electron microscope were conducted:

- Washing of the cells with cacodylate buffer (0.1 M) with 6.8 % Sucrose
- Fixation of 30' with 1 % glutaraldehyde
- Washing with cacodylate buffer
- Contrasting with 1 % Osmiumtetroxyde and 1 % potassium ferrocyanide for two hours
- Rinsing with cacodylate buffer for three times as well as with Aqua dest.
- Dehydration with increasing alcohol concentration: 20 %, 30 %, 50 %, 70 %, 90 %, 2x in 100 % EtOH for 10' each
- Embedding in Epon (epoxy resin), polymerisation for four days at 60 °C
- Ultramicrotomy, ultra-thin sections (70 nm; Leica Ultracut S, Leica Mikrosysteme GmbH, Bensheim, Germany)

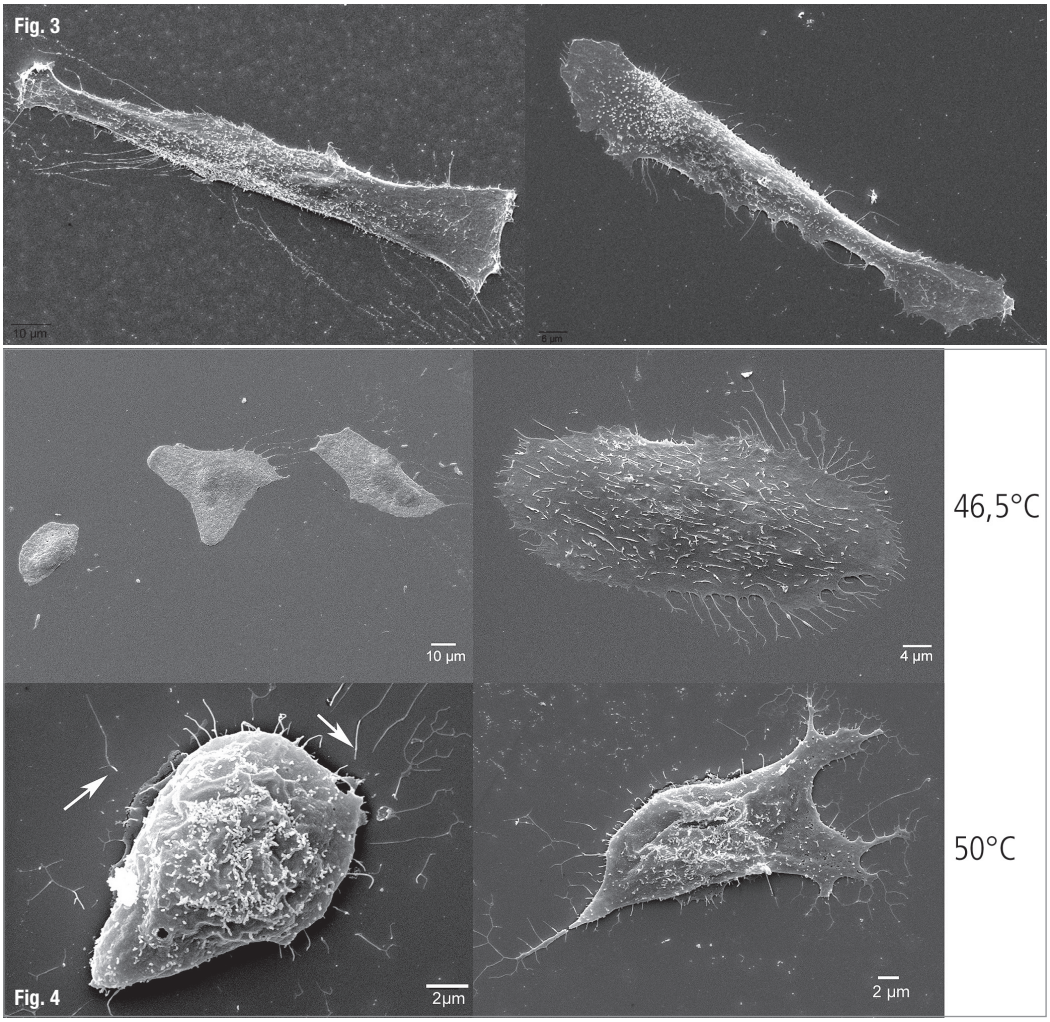


Fig. 3: Control cells exhibited a normal appearance at 37 °C under REM. Cell processes, microvilli-like structures on the cell surface (their numbers seems to depend on the level of cell activity) as well as the elongated cell shape are clearly visible.

Fig. 4: REM: Thermally treated DPSC showed external signs of cellular damage at 46.5 °C: The cell usually changes its elongated shape and starts to round. At 50 °C, an increased rounding can be observed. The cell seems to contract so fast that a part of the cytoplasm processes tears off (arrows). The surface structure of the cells is effected as appearance and number of microvilli change.

- Dyeing of the sections with 1 % Uranyl acetate in methanol and 1 drop of acetic acid for 10'
- Microscope: Zeiss EM 906.

Results

Light microscopy and vitality test

The cells received thermal treatment at temperatures ranging from 37 °C to 60 °C and varying intermediate temperature levels. Light microscopy examinations showed significant morphological changes at temperatures from 46.5 °C ± 0.5 °C.

At temperatures from 37 °C to 45 °C, all cells exhibited a green calcein fluorescence. At temperatures of 46 °C and above, lethal results were detected in some of the cells that had undergone thermal treatment. The number of lethal cells increased in correspondence to a rise in temperature.

At temperatures of 46 °C to 56.5 °C, the number of lethal cells had almost doubled 24 h after thermal treatment in comparison to the number of lethal cells one hour after thermal treatment (Table 1, Fig. 1). Starting at 56.5 °C, this phenomenon ceased, with

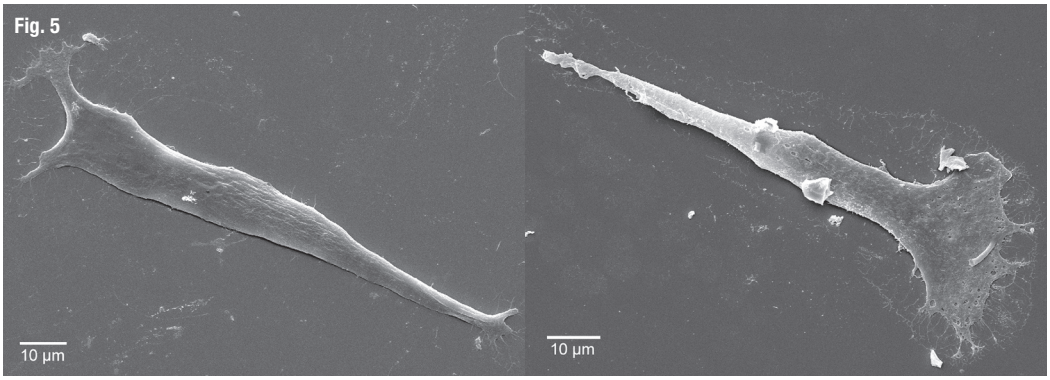


Fig. 5: REM: Thermal treatment at 60 °C. While the exterior shape remains mostly intact, their surface does not exhibit any structuring anymore.

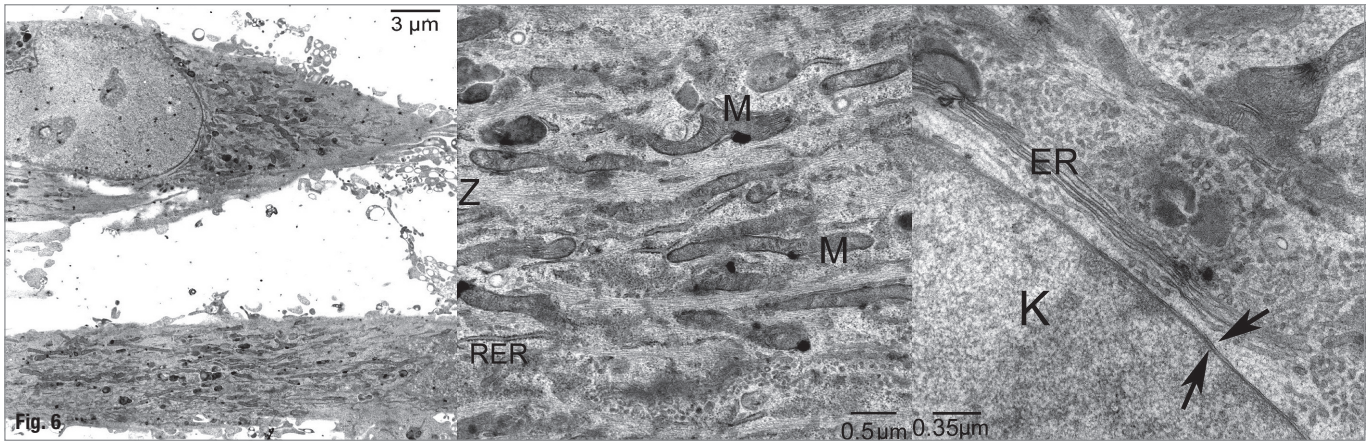


Fig. 6

about the same number of lethal cells. This temperature of 56.5 °C corresponded to the LD50 value (50 % lethality). No cell survived thermal treatment at 58 °C.

HSP production

Examinations with regard to the production of HSP via light microscope or transmission laser microscopy showed a slight, unspecific colouring of the cells after incubation of 37 °C (control, Fig. 2). An increase in HSP production (intense colouring) was noted at a temperature of 50 °C, while thermal treatment at 60 °C again resulted in slight, unspecific colouring of the cells.

REM

Scanning electron microscopy showed a typical flat, long distribution of the control cells (37 °C cells, Fig. 3). These cells exhibited many processes and microvilli-like structures. In addition, cell-to-cell connections with neighbouring cells were observed.

The successive rise in temperature resulted in the first critical temperature level of 46.5 °C ± 0.5 °C. From this level onwards, significant initial changes of the cells were registered via light and electron microscope, especially an initial deformation and rounding of the cells. The cell structure (microvilli-like structures) was reduced. However, microvilli were observed at temperatures of up to 50 °C (Fig. 4). At 50 °C (chance of survival > 70 °C according to Life/Dead Assay), the cells left distinct cytoplasm protuberances on the base of the coverslip (Fig. 4, arrow), probably caused by a rapid contraction or rounding.

Incubation at a temperature of 60 °C, at which none of the cells survived, resulted in a different outcome. There was no apparent deformation or rounding of the cells, with the original cell shape remaining mostly intact and some small reductions. The cells appeared to have been "thermally fixed" instantly. Neither microvilli nor other surface structures were visible. Cell processes in contact with the coverslip remained intact, but exhibited denaturation and fixation caused by rapid heating (Fig. 5).

TEM

The fibroblast-like DPSC cells (Fig. 6) exhibited long, extended mitochondria (M) within the 3-D network of the cell at 37 °C (control). The nucleus (K) appeared to be undivided and to have a normal nuclear envelope (arrows). ER/RER, free ribosomes as well as the Golgi apparatus did not show any anomalies. A significantly expressed cytoskeleton (Z) whose filaments were aligned parallely to the longitudinal axis (probably microfilaments) was observed. The cells featured a number of inclusions.

At 50 °C, cell rounding became irreversible (Fig. 7). Mitochondria (M) exhibited structural changes, especially an inflation which concurred with the destruction of the christae alignment, the parallelism of which got lost. There was no longer a three-dimensional network. The Golgi apparatus was significantly deformed and hardly any vesicles were constricted. The cytoskeleton was partially disintegrated and could no longer be detected. The cell membrane appeared to have increases vacuolisation. The nucleus (K) appeared to be damaged irreversibly. The nuclear envelope was inflated and partially disintegrated (*). The nuclear plasma condensed at the chromatin, resulting in a reduction of the euchromatin-areas which condensed at the heterochromatin. The nucleus exhibited segmented chambering (arrow).

Contrarily, the external shape of DPSC cells incubated at 60 °C (Fig. 8) remained mostly intact. However, cytoplasm was hardly detectable. Mitochondria (M) were destroyed, membranes and christae were partially wound up (arrows). Golgi apparatus and cytoskeleton were not detected. The euchromatin areas were reduced at the nucleus (K) and condensed at the heterochromatin (*). The nuclear membrane was significantly vesiculated.

Discussion

The first indications to a temperature-related damage of the DPSC were seen in the Life/Dead Assay.

Fig. 6: TEM: Control cells at 37 °C. K: Nucleus; ER: endoplasmatic reticulum, RER: rough endoplasmatic reticulum; M: mitochondria; Z: cytoskeleton; arrows: markers of the nuclear membrane.

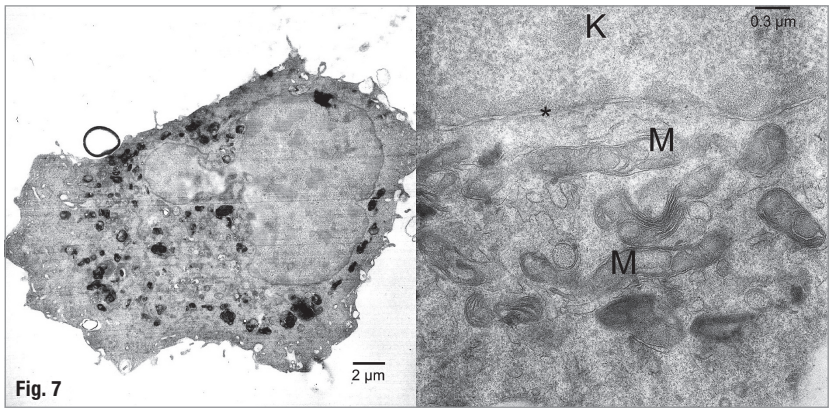


Fig. 7

Fig. 7: TEM: Due to thermal treatment at 50 °C, the cells are rounded and the cell membrane forms vesicles (left). Mitochondria exhibit a disrupted structure of the christae, while the nuclear plasma starts to condense and the nucleus (K) itself often appears to be uncharacteristically flapped (arrow). The nuclear membrane (*) seems to be partially inflated or dissolved.

Calcein is able to penetrate the membrane and is only converted to a fluorescent colouring agent inside of an intact cell. If the cell membrane becomes permeable as a result of damages, calcein will not remain inside the cell. As a consequence, Ethidium-homodimer-D1 will enter the cell in exchange. This substance is not permeable for intact membranes and will fluoresce red when combined with DNA.

Interestingly significant thermally-induced damages were only observed at temperatures ranging from 46.5 °C ± 0.5 °C. Starting at this temperature, cell membranes are destroyed apparently. Temperatures from 56.5 °C ± 0.5 °C form another threshold at which the 50 % lethality limit was reached.

If the vitality test was conducted 24 h after thermal treatment, almost twice as much lethal cells as observed 1 h after incubation were seen at temperatures from 46.5 °C to 56.5 °C. It appears that repairing processes cannot eliminate the thermal damage. Contrarily, thermal treatment will result in a lethal reaction even 1 h later.

Starting at 56.5 °C, most cells died immediately, probably due to denaturation of the proteins (coagulation). Usually, a temperature level of 62 °C is given as the starting point for coagulation in the literature.

However, the Life/Dead Assay does not allow any conclusions on the effects of the damages on the cell organells, compartments or physiological reactions such as protein production. Consequently, HSP tests and electron microscopic examinations of the ultrastructure were conducted additionally.

Heat-Shock-Proteins (HSP) were detected very well at 50 °C by an antibody reaction. The cells were distinctly coloured, which implies a significant reaction of the cell on the temperature-related stress. These cells were still able to synthesise the proteins and to survive for some time. Controls only showed only a light colouring, which may be the result of an unspecific reaction of the antibody with different cell proteins as well as a production of HSP which is not related to thermal stress.

Similarly, a temperature level of 60 °C only lead to light colouration, which can be explained by the immediate lethal effect resulting in a missing time scale for the biosynthesis of HSP. In general, it should be noted that the first HSP examinations did not exhibit the expected intracellular resolution due to a low specificity.

The results of REM and TEM at the different guide values of 37 °C, 46.5 °C, 50 °C, 60 °C and 65 °C fit very well with the results from light microscopy. The effects of a sudden and massive heating to more than 46 °C on the exterior cell shape (rounding and partial reduction of external structures) are distinctly visible. The extremely fast contraction of the cells at temperatures around 50 °C might result in the observed tearing of cytoplasm-processes. Thermally-related membrane openings were not detected via REM even at temperatures of 60 °C and above. These high temperatures probably resulted in an immediate coagulation of membrane proteins and other intracellular proteins, which lead to a "conservation" or fixation of the cells in their current shape. While the external cell shape was maintained because of the lacking time window for morphological modification, irreversible damages

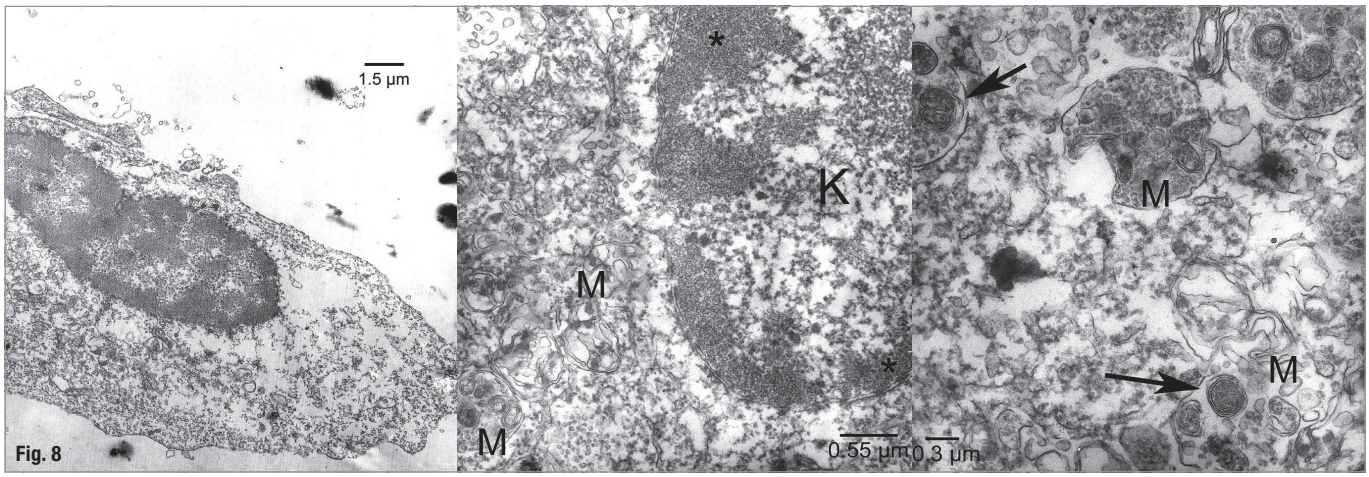


Fig. 8

Fig. 8: TEM: Thermal treatment at 60 °C. Parts of the cytoplasm are damaged or dissolved as can be seen by the mitochondria (M) with inflated or wound-up christae (arrows). The nucleus (K) shows severely condensed areas (*).

to the organells, nuclear membranes, nuclei and cytoplasm were detected electron-microscopically.

Starting at a temperature of 46.5 °C, a vacuolated cell membrane was observed via TEM in the rounded cells. Nucleus, organells and cytoskeleton were subject to beginning morphological changes.

The cells reacted differently on heating, probably because their differences in physiological age, activity and cycle states influenced immediately visible effects. For example, the cells differed in the level of microvilli reduction.

If the survival of thermally treated cells will prevail for a time span of more than 24 h and if there are thermally-related damages of the reproductive behaviour remains to be examined by further studies. However, it may be postulated with caution that the presented data indicate a chance of survival of the examined DPSC up to a temperature of 46 °C. These results on the thermal damage behaviour of human dental pulp stem cells are important for the development of ultrashort dental laser systems._

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Kurz & bündig

Es soll der Einfluss von Temperaturen im Bereich von 37 °C bis 65 °C auf Änderungen der Morphologie mittels licht- und elektronenmikroskopischer Aufnahmen, der Synthese von Heat Shock Proteinen (HSP) mittels fluoreszenzmarkierter Antikörper und der Vitalität mittels Life/Dead-Fluoreszenzkit von DPSC Stammzellen untersucht werden.

DPSC wurden bei 37 °C und 5 % CO_2 in sterilen Zellkammern (MiniCeM, JenLab GmbH, Jena) kultiviert. Für die Temperaturversuche wurden die Zellen mit vorgewärmtem Kulturmedium (Eagle's MEM, Gibco BRL, Paisley, Scotland, 37 °C) mit 20 % FCS, 2 mM L-Glutamin und 100 µM L-Ascorbat-2-Phosphat gespült, um Zelldebris zu entfernen. Anschließend wurde die Kammer mit Medium aufgefüllt und in ein vortemperiertes Wasserbad unterschiedlicher Temperatur eingebracht. Bis zu einer Inkubationstemperatur von 46 °C fanden die Versuche in Temperaturschritten von 2 °C statt, im sensiblen Bereich (46 °C – 58 °C) in Schritten von 0,5 °C. Zudem fanden Versuchsreihen bei 60 °C und 65 °C statt. Nach insgesamt 15 min Wärmebehandlung wurden die Zellen im Brutschrank bei 37 °C langsam für 1 Stunde heruntergekühlt.

Ein Teil der wärmebehandelten Zellen wurden nach Inkubation mit einem Life/Dead-Fluoreszenzassay (Molecular Probes, Eugene, OR, USA) zur Überprüfung der Vitalität fluoreszenzmikroskopisch mittels Axiovert 200 (ZEISS, Jena) untersucht. Ein Gemisch aus 2 µM Calcein AM und 4 µM Ethidium-homodimer-1 (EthD-1) wurde entweder 1 Stunde oder vergleichend 24 Stunde nach der Wärmebehandlung zu den langsam bei 37 °C im Inkubator abgekühlten Zellen in die Zellkammern gegeben und für 10' inkubiert. Vitale Zellen zeigten durch das Calcein eine grüne Fluoreszenz, letale Zellen eine rote Kernfluoreszenz (Ethidium-homodimer-1 an DNA gekoppelt). Es wurden jeweils 100 Zellen ausgezählt.

Die Zellen reagierten etwas unterschiedlich auf die Erwärmung, möglicherweise nahmen ein unterschiedliches physiologisches Alter und verschiedene Aktivitäts- und Zyklusstadien einen Einfluss auf die sofort sichtbaren Effekte, z. B. war die Reduktion der Mikrovilli nicht überall gleich stark ausgebildet.

Ob das Überleben der wärmebehandelten Zellen über einen größeren Zeitraum als 24 Stunden gegeben ist und inwieweit thermisch bedingte Schäden des Reproduktionsverhaltens vorliegen, müssten weiterführende Studien untersuchen. Eine teilweise Überlebenschance der untersuchten DPSC bis zu einer Temperatur von 46 °C könnte aus den vorliegenden Daten jedoch vorsichtig postuliert werden.