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THE EFFECT OF EXERCISE-INDUCED LOCALISED HYPERTHERMIA ON TENDON CELL SURVIVAL

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Summary

Tendons that store energy during locomotion, such as the equine superficial digital flexor tendon (SDFT) and human Achilles tendon, suffer a high incidence of central core degeneration which is thought to precede tendon rupture. Although energy storage contributes to the efficiency of locomotion, tendons are not perfectly elastic and some energy is lost in the form of heat. Recent studies have shown that the central core of equine SDFT reaches temperatures as high as 45 °C during high-speed locomotion. In this study, we test the hypothesis that hyperthermia causes tendon cell death and results in tendon central core degeneration. Tendon fibroblasts cultured from the core of the equine SDFT were subjected to a temperature of 45 °C in an *in vitro* system for 0–180 min, and cell survival fraction was measured and compared with that for equine dermal fibroblasts and a commercial rat kidney fibroblast cell line (NRK 49F). Tendon fibroblasts were significantly more resistant to hyperthermia than NRK 49F cells after 30, 45 and 60 min of heating and significantly more resistant than dermal fibroblasts after 45 and 60 min of heating. After 10 min of heating at 45 °C, the tendon fibroblast cell survival fraction was 91±4 %, whereas heating for 10 min at 48 °C resulted in a drop in the cell survival fraction to 22±4 %. In conclusion, while temperatures experienced in the central core of the SDFT *in vivo* are unlikely to result in tendon cell death, repeated hyperthermic insults may compromise cell metabolism of matrix components, resulting in tendon central core degeneration.

Key words: exercise, hyperthermia, tendon, cell death, horse, cell biology, cell culture, cell proliferation, degeneration, tendon injury, tendon mechanics

Introduction

Tendons function as mechanical links, transmitting the force generated by a muscle to a distant bone and bringing about the movement of a segment of the limb. However, tendons in the legs and feet of many terrestrial animals also play a more specialised rôle by acting as elastic energy stores (Alexander, 1988). As the foot lands, tendons such as the equine superficial digital flexor tendon (SDFT) are stretched as the metacarpophalangeal joint hyperextends and sinks towards the ground. Kinetic energy is stored transiently as strain energy in the tendon. Elastic recoil then converts most of the stored energy back to kinetic energy as the foot leaves the ground. This important mechanism saves substantial quantities of muscular energy during locomotion (Alexander, 1988). The stored strain energy, however, is not all recoverable, owing to internal viscous damping, and some 5–10 % is released as heat (Ker, 1981; Riemersma and Schamhardt, 1985). Therefore, if the blood supply to the structure is not sufficient to dissipate the heat generated, a rise in temperature would be expected to occur.

Substantial temperature increases have indeed been shown to occur *in vivo* in the central core of equine superficial digital flexor tendons during high-speed locomotion. Temperatures as high as 45 °C have been recorded in the core of this tendon during gallop exercise, while the tendon surface temperature plateaued at 5 °C lower (Wilson and Goodship, 1994). Mathematical models, based on mechanical and other properties, suggest that similar rises occur in human Achilles tendon during vigorous exercise (Wilson and Goodship, 1994).

Mammalian cells from other tissues are known to tolerate increases in temperature rather poorly (Dewey *et al.* 1977). Severe alterations in the morphology of V79 fibroblasts are evident after exposure to a temperature of 45 °C (Arancia *et al.* 1989). The cells within tendons play an important rôle in maintaining the extracellular matrix through the synthesis of collagen and other matrix components. Therefore, damage to the cell components may also compromise the integrity of the extracellular matrix. Significantly, the central core of the tendon, which is the site of most marked temperature increases, is also the site of degeneration and subsequent injury in both the equine SDFT (Webbon, 1977) and human Achilles tendon (Arner *et al.* 1959). This suggests that exercise-induced hyperthermia may play a rôle in the pathogenesis of these degenerative core lesions.

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It thus seemed important to determine whether tenocytes display a similar sensitivity to hyperthermia to that of other fibroblast cell types. This study tests the hypothesis that exercise-induced hyperthermia in elastic storage tendons causes tendon cell death within the central core of these tendons, which would then result in localised tissue degeneration.

The effect of hyperthermia was examined on cells cultured from the core of the equine SDFT. As a comparison, experiments were repeated using equine dermal fibroblasts and a commercially available rat kidney fibroblast cell line (NRK 49F). Lethal cell damage following exposure to hyperthermia can be assessed by measuring the cell fraction that is able to re-establish under culture conditions. Other methods, such as Trypan Blue exclusion, are not as reliable as cells may appear to be viable immediately following exposure but subsequently are unable to survive. A temperature of 45 °C was chosen for the experiments as this was the highest temperature recorded consistently *in vivo*. The time of incubation was extended until the cell survival fraction fell to below 10 %. The period for which a horse would be expected to gallop during a competition at a speed great enough to produce temperature rises of any magnitude would not normally exceed 10 min. Further studies, therefore, were conducted where exposure to hyperthermia was limited to 10 min, but a range of temperatures from 37 to 52 °C was used.

Materials and methods

Cell culture

Superficial digital flexor tendons (Fig. 1) were collected at a local abattoir from half- to full-Thoroughbred horses (mean age 11 years, range 2–23 years, *N*=6) with no known history of clinically detectable tendon injury. Loose paratenon connective tissue was dissected away from the surface of the tendon in the unsheathed mid-metacarpal region, and a length of tissue (approximately 1 cm) was taken and washed thoroughly (Fig. 2).

Tissue fragments of approximately 1 mm^3 were cut from the centre of the tendon and placed in tissue culture grade plastic Petri dishes (60 mm diameter) at three per dish. These were covered with a sterile circular glass coverslip. DMEM (Dulbecco's modified Eagle's medium) (5 ml) supplemented with 20 mmol l^{-1} Hepes, 10 % FCS (fetal calf serum) plus antibiotics (streptomycin, 100 μg ml^{-1}; benzylpenicillin, 200 i.u. ml^{-1}) and fungizone (amphotericin, 5 μg ml^{-1}) was added to each dish, and the cultures were then incubated in a humidified incubator with 5 % CO_2 at 37 °C.

The explant-derived cells took approximately 1 week to appear from the tissue fragment and approximately another week to become confluent and cover the coverslip. Once this had occurred, coverslips were inverted and the medium and explants removed. Cells were then released with 0.05 % trypsin in sterile phosphate-buffered saline (PBS) containing 0.02 % EDTA and Phenol Red (0.01 %) and transferred in DMEM (5 ml) supplemented with 20 mmol l^{-1} Hepes, 10 % FCS plus antibiotics to 25 cm^2 tissue culture flasks. When cells reached confluence, they were transferred to successively larger flasks and finally maintained in monolayer in 175 cm^2 flasks in DMEM supplemented as above.

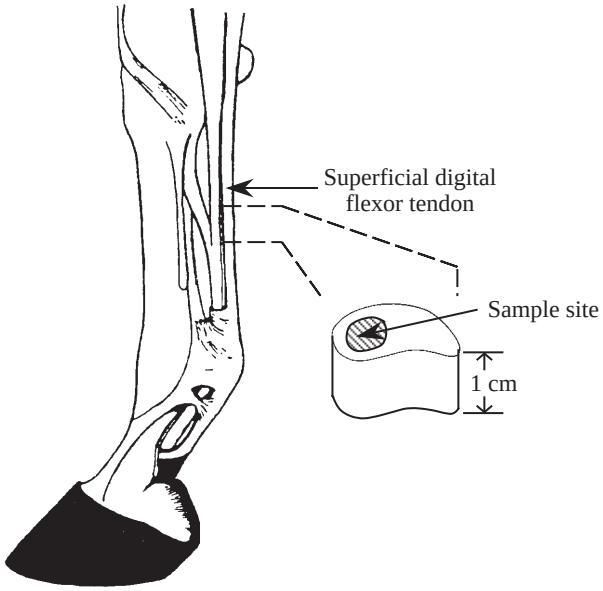

Fig. 1. Distal part of the equine forelimb showing tissue origin.

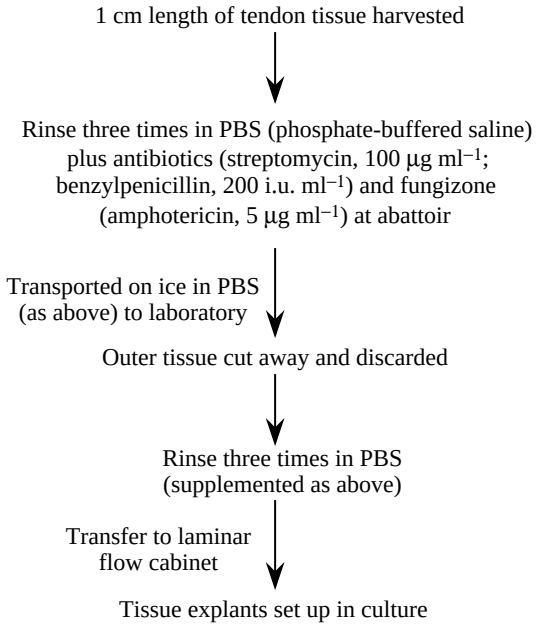

Fig. 2. Protocol followed for tissue washing to ensure sterility.

Explant-derived cells had the characteristic appearance of fibroblasts; cell processes protruded in a star-like shape in sparse cell cultures, and as confluence was reached cells became spindle-shaped, producing a parallel array.

Dermal fibroblasts were grown in exactly the same way as the tendon fibroblasts from explants of equine skin (*N*=4)

taken from the region overlying the SDFT. In addition, a commercially available fibroblast cell line, NRK 49F (Flow Laboratories, Hertfordshire, UK), was cultured under the same conditions.

Exposure to hyperthermia for different periods

Cells between passages 3 and 8 were grown to confluence and then released from the surface of the flask with trypsin (as described above). Cells were washed in medium and resuspended in culture medium supplemented as above to give approximately 0.5×10^6 cells ml^{-1}. Samples of cell suspension (1 ml) were immersed in a waterbath at 45 °C in 7 ml tubes for 0–180 min. After heating, all tubes were returned to a waterbath set at 37 °C for 5 min until the medium had returned to 37 °C. The time taken for the cell suspension to equilibrate with the waterbath temperature was determined in previous experiments by inserting a temperature probe into the tube (Fig. 3). The rate of temperature rise was similar to the rate of temperature rise in the core of the tendon *in vivo* (Wilson and Goodship, 1994). After heating and re-equilibrating at 37 °C, cells were transferred back into culture flasks, fresh medium supplemented as above was added, and the flasks were returned to the 37 °C incubator for 24 h, during which time viable cells were able to re-adhere to the surface of the flask.

Exposure to hyperthermia of varying intensities

Tendon fibroblasts, dermal fibroblasts and NRK 49F cells were heated for a fixed period of 10 min at a range of temperatures from 37 to 52 °C in the same way as described above.

Quantification of cell survival

Viable cells adherent to the bottom of the flask were quantified by a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Plumb *et al.* 1989). The MTT assay is specific for viable cells and showed a very good correlation with cell number (Fig. 4). Medium and dead cells were decanted, and fresh medium containing MTT (0.6 mg ml^{-1}) was added. Flasks were returned to the incubator at 37 °C for 1 h. After this time, the medium was decanted and the precipitate dissolved in 2 ml of DMSO. Sorenson's buffer at pH 10.1 (0.625 ml) and H_2O (8 ml) were added and the absorbance read at 570 nm on a spectrophotometer. A blank flask was also prepared in exactly the same way except that no cells were present. Results were expressed as percentage cell survival relative to those cells kept at 37 °C (i.e. 0 min at 45 °C). All data are given as mean ± S.E.M.

Statistical analysis

Statistical significance was evaluated using analysis of variance (ANOVA) and, where a statistical difference was detected, *post hoc* Student's *t*-tests were used to determine differences between individual groups.

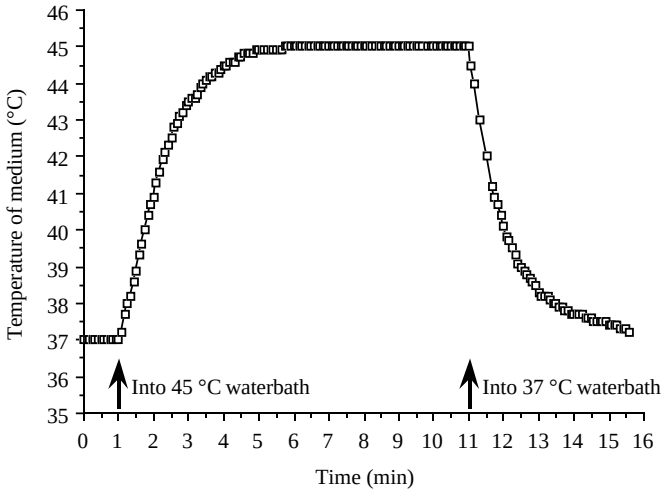

Fig. 3. Time taken for a cell suspension to equilibrate with waterbath temperature.

Results

Exposure to hyperthermia for different periods

The percentage of cells surviving following exposure to a temperature of 45 °C for 0–180 min is shown in Fig. 5. NRK 49F cells showed a sharp decline in cell viability after 10 min of heating. Tendon fibroblasts showed a more gradual decline in viability and were significantly more resistant to hyperthermia than NRK 49F cells at 30 min (P=0.002), 45 min (P<0.001) and 60 min (P<0.001). Dermal fibroblasts were also more resistant to hyperthermia than NRK 49F cells at 30 min (P=0.04), 45 min (P=0.003) and 60 min (P=0.04), but significantly more sensitive to 45 °C than tendon fibroblasts after heating for 45 min (P=0.01) and 60 min (P=0.02). After 1 h of exposure to 45 °C, the percentage of tendon fibroblasts remaining viable was 51.6±5.5 % compared with 25.6±7.5 % for dermal fibroblasts and 5.7±1.2 % for NRK 49F cells. Following exposure to 45 °C for 120 and 180 min, cell survival

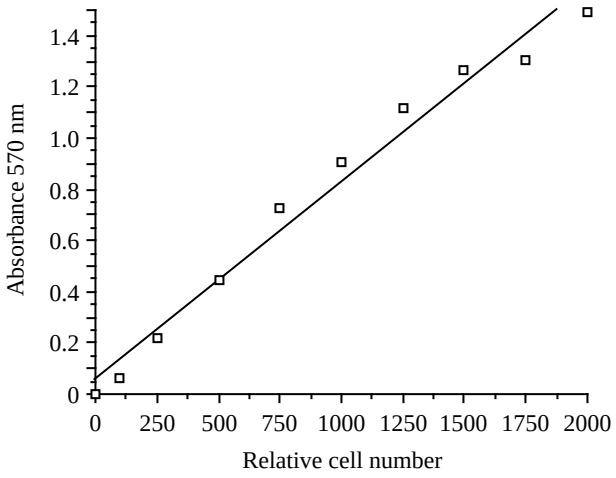

Fig. 4. Relationship between MTT–formazan production (measured as absorbance at 570 nm) and cell number: y=0.000767x+0.056897, r^2=0.98, P<0.001, for this experiment.

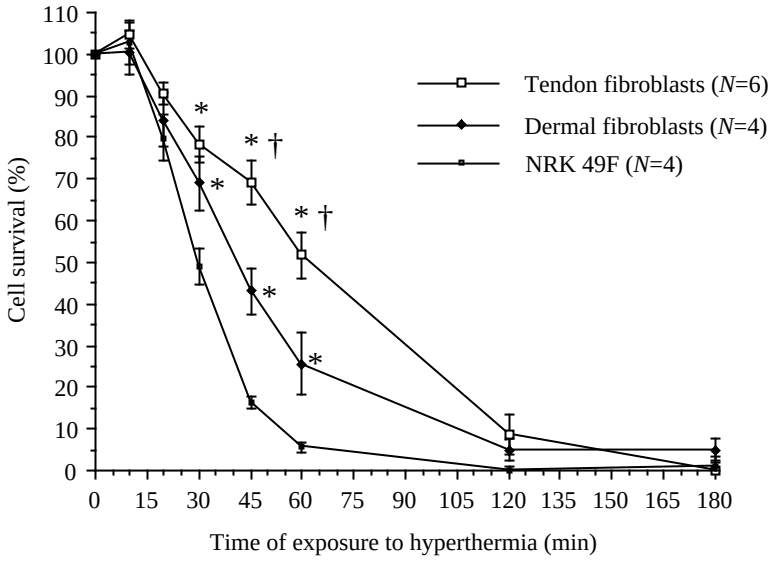

Fig. 5. Cell survival following exposure to 45 °C. Data are presented as mean ± S.E.M. * denotes a significant difference from NRK 49F cells and † a significant difference from dermal fibroblasts (P<0.05).

had dropped to below 10 % and there was no significant difference between cell lines.

Exposure to hyperthermia of varying intensities

When heated for 10 min, the critical temperature at which substantial cell death occurs appears to be between 46 and 48 °C for both tendon and dermal fibroblasts (Fig. 6). There were no significant differences between these two cell lines when the temperature was varied but the time of exposure held constant at 10 min. NRK 49F cells, however, showed a sharp decline in viability at a temperature of 46 °C and were significantly more sensitive than equine tendon and dermal fibroblasts at temperatures of 46 °C (P≤0.01), 47 °C (P<0.001) and 48 °C (P<0.001).

Discussion

The results presented in this study suggest that the elevated temperature induced in the core of the tendon by high-speed locomotion does not persist for long enough to cause tendon cell death *in vivo*. A racehorse would not be capable of sustaining a fast gallop for longer than approximately 10 min. It therefore does not seem likely that cell death *per se* is an explanation for tendon central core degeneration. However, repeated exposure to short periods of hyperthermia may compromise cell function, resulting in a reduced synthetic capability or a change in cell metabolism which may alter matrix composition. These results are compatible with previous observations that cellularity is not significantly reduced in the central core of degenerated tendons but that changes in matrix composition are evident (Birch, 1993).

The MTT assay used in these experiments gives a relatively immediate assessment of cell viability. It is possible that reproductive integrity will be lost, the effect of which would become apparent in the longer term. This may explain why other cell lines, when assayed by colony formation several

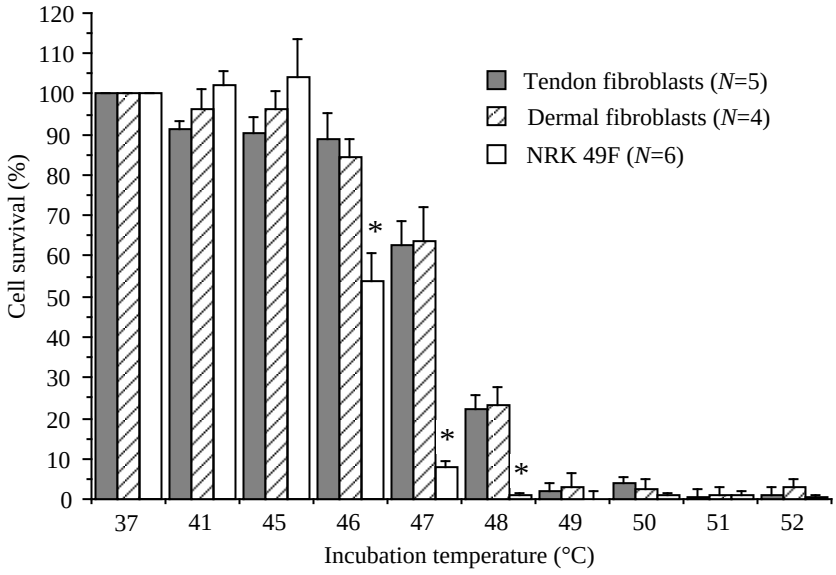

Fig. 6. Cell survival following 10 min of exposure to hyperthermia. Data are presented as mean + S.E.M. * denotes a significant difference from tendon and dermal fibroblasts (P<0.05).

days after insult, appear to be more sensitive to hyperthermia than the cell lines used in these studies (Dewey *et al.* 1977).

A temperature of 45 °C was chosen for the initial experiment as this was the highest temperature recorded in the *in vivo* studies of Wilson and Goodship (1994). To record peak temperatures in their studies, horses were galloped at a speed of $10\,m\,s^{-1}$ and did not carry a rider. An elite racehorse, however, would be able to gallop at a greater speed than this and, in addition to the weight of a jockey, this would result in higher tendon strains. Thus, it is possible that in some instances a peak temperature greater than 45 °C may be reached in the core of the tendon. The results obtained in the second part of this study demonstrate that a rapid decline in tendon fibroblast viability occurs after 10 min of exposure to temperatures between 46 and 48 °C. These results suggest that in exceptional circumstances cell death may possibly occur as a result of high-speed locomotion.

Heating cells in an *in vitro* system as carried out in these studies is not completely analogous to the situation *in vivo*. There are several important differences which may influence cell viability. Tendon cells growing in culture are devoid of the matrix in which they are embedded *in vivo*. Matrix macromolecules interact specifically with the cell *via* cell surface receptors, such as integrins, which communicate with the cell cytoskeleton; in this way, extracellular macromolecules are able to influence cell metabolism (Labat-Robert *et al.* 1990). In these experiments, cells were heated whilst maintained in suspension. Cells that are attached to a solid support are flattened and contain a more extensive and complex cytoskeleton than unattached rounded cells (Ben-Ze'ev, 1987; Ungar *et al.* 1986). It has been shown that an intact cytoskeletal system is more sensitive to inactivation by heat than its unassembled proteins (Coss *et al.* 1982). Correspondingly, cells heated in suspension are less sensitive to heat killing than cells treated as monolayers (Smith *et al.* 1993).

Cells grown in culture are exposed to mitogenic factors present in the fetal calf serum which cause the cells to proliferate and progress through the cell cycle. Tendon cells *in vivo*, however, are not rapidly dividing and thus enter a specialised resting state known as G_0. The cell cycle phase has been found to have an effect on cell sensitivity to hyperthermic insults. The fraction of cells surviving when heated in the S phase is lower than that for cells heated in the G_1 phase (Wachsberger and Coss, 1990). In addition, cells heated in G_1 or S phase appear to die by different mechanisms, emphasizing the need to use synchronous populations of cells. In our experiments, cells were grown to confluence where cell-to-cell contact inhibits proliferation and cells are arrested in the G_1 phase of the cycle.

Other factors which have previously been implicated in contributing to exercise-related damage of tendons include tissue ischaemia, which may lead to hypoxia (Fackelman, 1973). This is because, during locomotion, the SDFT is stretched and released and this cyclical loading may intermittently cut off the blood supply to the tendon. As we have recently demonstrated, cells from the mature SDFT possess oxidative enzymes and show a dependence upon oxidative metabolism for the maintenance of intracellular ATP levels (Birch *et al.* 1997). In addition, poor blood supply may result in nutrient deprivation. Cells that are nutrient-deficient and/or at low pH are more sensitive to killing by heat (Hall and Roizin-Towle, 1984). However, cells heated *in vitro* are provided with all the essential nutrients in the culture medium and the pH is held constant by buffering. Therefore, both hypoxia and nutrient deprivation *in vivo* may increase the sensitivity of cells to hyperthermic damage, thereby making cells *in vivo* more sensitive to damage than those *in vitro*.

The cell survival fraction in these experiments cannot therefore predict directly what might occur *in vivo* during high-speed locomotion because of the differing conditions mentioned above. It is, however, of biological significance that equine tendon fibroblasts are more resistant to hyperthermic damage than equine dermal fibroblasts grown and exposed to hyperthermia under exactly the same conditions. The thermal resistance of tendon fibroblasts may be due to adaptation following repeated exposure to elevated temperatures *in vivo* during high-speed locomotion or may represent an inherent genetic difference between cell types. It has been known for many years that different mammalian cell types can display a different sensitivity to killing by heat (Auersperg, 1966), although the mechanism bestowing thermal tolerance on some cell types is still not known. Indeed, it is not yet clear what causes heat-induced cell death; cellular membranes, cytoskeletal structures, energy metabolism, protein synthesis and DNA have all been suggested as possible targets (Steels *et al.* 1992). Exposure of cells to non-lethal temperatures evokes an immediate, but transient, response which enables them to survive subsequent exposure to temperatures which under normal conditions would be lethal (Donati *et al.* 1990). This response is known as the 'heat shock response', and heat shock proteins, in particular HSP70, play a definitive role in this phenomenon (Riabowol *et al.* 1988; Johnston and Kucey, 1988). Heat shock proteins are also present in unstressed cells, representing approximately 2–3 % of cellular protein (Donati *et al.* 1990), where their function appears to relate to 'chaperoning' and preventing incorrect folding of other proteins (Munro and Pelham, 1986). It may be, therefore, that tendon fibroblasts have higher basal levels of these proteins. Tolerance to heat stress, however, is not necessarily associated with the induction of heat shock proteins (Steels *et al.* 1992).

Cell number, as measured in these experiments, depends on the balance between cell proliferation rate and the rate of cell death. In these experiments, it is not possible to distinguish between several alternative explanations for the difference in cell number 24 h after the heat insult. First, it may be that a greater number of dermal and NRK 49F fibroblasts than tendon fibroblasts undergo cell necrosis following exposure to hyperthermia. Second, an equal number of tendon, dermal and NRK 49F cells may die, but the tendon cells may proliferate more rapidly, thus appearing to be more tolerant. Third, the rate of proliferation may remain unaltered, but the rate of

programmed cell death or apoptosis may increase in the dermal and NRK 49F fibroblasts. Further experiments need to be conducted to determine the effect of hyperthermia on cell cycle time and the rate of apoptosis.

In conclusion, temperatures experienced in the central core of the SDFT *in vivo* are unlikely to result in tendon cell death. Tendon central core degeneration may, however, be due to a change in the metabolism of extracellular matrix components in this zone as a result of repeated exposure to sublethal thermal insults. Tendon fibroblasts appear to have an inherent thermal tolerance compared with dermal fibroblasts and NRK 49F cells. The mechanism bestowing thermal tolerance on tendon fibroblasts has yet to be determined.

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