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The Effect of Dehydrothermal Treatment on the Mechanical and Structural Properties of Collagen-GAG scaffolds[3]

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Abstract

The mechanical properties of a tissue engineering scaffold are critical for preserving structural integrity and functionality during both *in vivo* implantation and long-term performance. In addition, the mechanical and structural properties of the scaffold can direct cellular activity within a tissue-engineered construct. In this context, the aim of this study was to investigate the effects of dehydrothermal (DHT) treatment on the mechanical and structural properties of collagen-glycosaminoglycan (CG) scaffolds. Temperature (105–180°C) and exposure period (24–120 h) of DHT treatment were varied to determine their effect on the mechanical properties, crosslinking density and denaturation of CG scaffolds. As expected, increasing the temperature and duration of DHT treatment resulted in an increase in the mechanical properties. Compressive properties increased up to 2-fold, while tensile properties increased up to 3.8-fold. Crosslink density was found to increase with DHT temperature but not exposure period. Denaturation also increased with DHT temperature and exposure period, ranging from 25% to 60% denaturation. Crosslink density was found to be correlated with compressive modulus, whilst denaturation was found to correlate with tensile modulus. Taken together, these results indicate that DHT treatment is a viable technique for altering the mechanical properties of CG scaffolds. The enhanced mechanical properties of DHT-treated CG scaffolds improves their suitability for use both *in vitro* and *in vivo*. In addition, this work facilitates investigation of the effects of mechanical properties and denaturation on cell activity in a 3D environment.

Keywords: Tissue Engineering, Crosslinking, DHT, Denaturation, FT-IR

INTRODUCTION

Collagen-glycosaminoglycan (CG) scaffolds were originally developed for skin regeneration and have shown clinical success in this area.[1-4] More recently, CG scaffolds have shown great potential in tissue engineering of bone, cartilage and nerve.[5-7] Various forms of collagen have been used clinically, including aqueous injectable collagen, powders, surgical sutures, corneal shields and spongy implants.[8] Collagen is particularly attractive for tissue engineering due to its excellent biocompatibility, degradation into physiological end-products and suitable interaction with cells and other macromolecules.[9] Despite these advantages, CG scaffolds have limitations for use in tissue engineering, most notably low mechanical properties. The mechanical properties of scaffolds for any tissue engineering application are important as the scaffold must have appropriate characteristics in order to facilitate *in vivo* implantation and to be mechanically functional once implanted. Mechanical properties are also important for applying the correct biomechanical stimuli to cells within the scaffold; for example, substrate elasticity has been shown to influence stem cell differentiation, adhesion and growth in 2D.[10-13] Therefore, improving the mechanical properties could vastly improve the range of applications for CG scaffolds in tissue engineering. In addition, the ability to fabricate scaffolds with a range of mechanical properties is needed to study how scaffold elasticity affects cellular activity in a 3D environment.

The mechanical properties of CG scaffolds can be altered by varying the degree of crosslinking between collagen fibres. Crosslinks are bonds between side chains of amino acids present in collagen molecules. These bonds increase the stiffness of collagen fibres

by preventing the long rod-like collagen molecules from sliding past each other under stress.[14] Dehydrothermal (DHT) treatment is a common technique for stabilising collagen and collagen composite materials.[2,15,16] It is a physical treatment that involves subjecting collagen to increased temperature (>90°C) while under vacuum. This removes water from the collagen molecules, resulting in the formation of intermolecular crosslinks through condensation reactions either by esterification or amide formation.[17] DHT treatment is favourable to other crosslinking methods as it does not involve the use of cytotoxic reagents. A further advantage of DHT treatment is the sterilisation provided by the high temperatures and exposure periods used. Previous studies on DHT treatment have shown that increasing DHT temperature and exposure duration improves the mechanical properties of collagen fibres.[18,19]

In addition to forming crosslinks, the high temperatures used during DHT crosslinking have been shown to denature collagen.[20] Denaturation is defined as rearrangement of the triple helix into a random chain configuration.[21] While generally considered undesirable as it disrupts the native collagen conformation, denaturation may reduce the inflammation response and increase cellular infiltration *in vivo*.[22] A greater knowledge of the level of denaturation after DHT treatment will enable further investigation of the relationship between cellular activity and denaturation.

Despite the widespread use of DHT treatment on collagen films, fibres and scaffolds, a detailed analysis has not been carried out to determine how the process parameters affect CG scaffold parameters. Therefore, the specific objectives of this study were to: 1)

determine the range of compressive and tensile properties achievable using DHT by varying temperature and exposure period, 2) to evaluate the level of crosslinking after DHT treatment, 3) to establish the level of denaturation after crosslinking, and 4) to correlate changes in mechanical properties with crosslinking and denaturation.

MATERIALS AND METHODS

Scaffold fabrication

Scaffolds were produced by freeze-drying a collagen-GAG slurry.[23] To prepare the slurry, type I bovine collagen (Integra Life Sciences, Plainsboro, NJ), chondroitin-6-sulphate (Sigma-Aldrich Chemical Co., St. Louis, MO) and 0.05 M glacial acetic acid were blended together at 15,000 rpm using an overhead blender (Ultra Turrax T18, IKA Works Inc., Wilmington, NC). Blending was carried out in a reaction vessel, which was maintained at 4°C using a circulation cooling system (WKL 230, Lauda, Germany). The resulting collagen-GAG slurry contained 0.5% (w/v) collagen and 0.044% (w/v) chondroitin-6-sulfate. The slurry was then degassed in vacuum desiccator for 60 min to remove air bubbles from the solution.

The slurry was freeze-dried as described previously.[23] Briefly, 67.25 ml of the collagen-GAG slurry was pipetted into a stainless steel pan (5 x 5 in, grade 304 SS). The tray was placed onto the freeze-dryer shelf (Advantage EL, VirTis Co., Gardiner, NY) and cooled to -40°C at 0.9°C/min. Previous work has found that this freezing protocol produces scaffolds with a mean pore size of 96 μm.[6,23] Once freezing was complete, the ice crystals

were removed via sublimation for 17 h at 0°C and 200 mTorr. This process produces a highly porous sheet of CG scaffold.

DHT treatment

DHT treatment was carried by placing the scaffolds in an aluminium foil packet inside a vacuum oven (Vacucell 22, MMM, Germany) under a vacuum of 0.05 bar. To determine the effect of DHT parameters on CG scaffold properties, exposure period and crosslinking temperature were varied. Exposure period was varied from 24 h to 120 h, at 24 h intervals, and four crosslinking temperatures were used: 105°C, 120°C, 150°C and 180°C.

Mechanical testing

Compressive and tensile testing were used to determine the effect of DHT parameters on the mechanical properties of the scaffolds. Mechanical testing of scaffold samples was carried out using a mechanical testing machine (Z050, Zwick/Roell, Germany) fitted with a 5-N load cell. Samples were pre-hydrated in phosphate buffered saline (PBS) for one hour prior to testing and all testing was carried out in a bath of PBS. For unconfined compression testing with impermeable, un-lubricated platens, samples of 8 mm diameter were cut from the scaffolds using a punch. For tensile testing, rectangular strips (10 x 40 mm) were cut from the scaffolds using a razor blade. The gauge length used for tensile testing was 25 mm. Both tensile and compressive testing were conducted at a strain rate of 10%/min. The modulus was defined as the slope of a linear fit to the stress-strain curve over 2-5% strain.

Fourier transform infra-red microscopy

Fourier transform infra-red (FT-IR) spectra were used to investigate the level of crosslinking and collagen denaturation after DHT treatment.[20,24,25] FT-IR microscopy was carried out using a Spectrum One FT-IR (Perkin Elmer, UK). Cylindrical samples (8 mm diameter) were used for the analysis. Spectra were analysed using a free-ware program, Spekwin (Daltrozzo Group, University of Konstanz, Germany). Formation of crosslinks through DHT treatment can be monitored by analysing the amide II band absorbance peak at 1553 cm^{-1}.[26,27] The band at 1553 cm^{-1} is proportional to the amount of NH_2, which is converted to NH during the formation of crosslinks through condensation reactions.[28] Therefore, a decrease in the absorbance at 1553 cm^{-1} corresponds to an increase in the number of crosslinks. The absorbance at 1553 cm^{-1} was normalised to the absorbance at 1450 cm^{-1} (1553 cm^{-1}/1450 cm^{-1}), which is unaffected by the formation of crosslinks or denaturation.[25,27] This inverse of this ratio was defined as the effective crosslink density.

The absorbance peak at 1235 cm^{-1} (waves per cm) is affected by changes in the triple helical structure of collagen and can therefore be used to quantify the denaturation of a collagen sample. The absorbance at 1235 cm^{-1} was normalised to the absorbance at 1450 cm^{-1}.[20,24,25] By measuring the absorbance of an untreated sample (0% denatured) and gelatine (collagen that has lost its triple helical structure and is therefore 100% denatured), linear interpolation was used to measure the percentage denaturation of a test sample.[20,24]

Statistical analysis

Results are expressed as mean ± standard deviation. Two-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Tukey test) was used to evaluate the effects of DHT exposure period and temperature on compressive modulus, tensile modulus, denaturation and crosslinking density. Multiple linear regression analysis was used to examine correlations between mechanical properties, crosslinking density and denaturation. Separate regressions for compressive and tensile moduli were carried out, with percentage denaturation and crosslink density set as the independent variables. Statistical significance was declared at $p \leq 0.05$. A correlation between tensile modulus and denaturation was found upon analysis. In order to further investigate this relationship a subset of the data was tested to failure in tension and the ultimate tensile strength and strain to failure were calculated.

RESULTS

Mechanical properties

Compressive modulus increased with increasing temperature but not with increasing exposure period (Fig 1 A). At 24 h, compressive modulus increased 2-fold for scaffolds crosslinked at 180°C compared to 105°C ($p<0.001$). Exposure period had no effect on compressive modulus at 105, 120 and 150°C ($p>0.05$). However, at 180°C the modulus decreased by 20% when the exposure period was increased beyond 24 h ($p<0.001$).

Tensile modulus increased with both increasing temperature and increasing exposure period (Fig 1 B). Tensile modulus increased 3.8-fold when temperature was increased from 105°C to 180°C ($p<0.001$). Increasing exposure duration had no effect on tensile modulus at 105°C ($p>0.05$), whereas at 120 and 150°C there was an increase in tensile modulus with increased exposure duration ($p<0.05$). At 180°C, the modulus decreased by up to 26% after 72 h ($p<0.001$).

Crosslink density

Crosslink analysis of DHT treated samples show that the level of NH_2 decreased with increasing temperature but not exposure period ($p<0.05$). Since NH_2 levels are inversely proportional to crosslink density, this demonstrated an increase in the crosslink density with increasing temperature but not exposure period (Fig. 2 A).[26]

Denaturation

FT-IR analysis showed that denaturation increased with both DHT exposure and temperature (Fig 2 B, $p<0.05$). Increasing exposure period had no effect on denaturation at 105 and 180°C ($p>0.05$). However, at 120 and 150°C denaturation increased with exposure period ($p<0.05$). Scaffolds contained 25% denatured collagen after treatment at 105°C for 24 h and 60% denatured collagen after extensive treatment at 180°C for 120 h.

Multiple linear regression

Multiple linear regression analysis was carried out to investigate correlations between elastic moduli, denaturation and crosslink density. Analysis showed the effect of

denaturation on compressive modulus was not significant, nor was the effect of crosslink density on tensile modulus. Therefore, these variables were removed in the relevant regressions and the regressions were subsequently simplified. The compressive modulus was found to be highly correlated with crosslink density (Fig.3 A, $p<0.001$, $R^2=0.70$). The tensile modulus was highly correlated with percentage denaturation (Fig.3 B, $p<0.001$, $R^2=0.85$). As tensile modulus was correlated with denaturation, samples at three levels of denaturation, (25, 40 and 60% corresponding to DHT treatments of 105°C for 24 h, 150°C for 48 h and 180°C for 48 h respectively) were tested to failure in tension. There was a significant decrease in UTS with increasing denaturation (Fig. 4 A, $p<0.05$). Strain to failure also decreased significantly with increasing denaturation (Fig. 4 B, $p<0.001$).

DISCUSSION

The mechanical properties of scaffolds for tissue engineering are critical for preserving structural integrity and functionality during both *in vivo* implantation and long-term performance. In addition, the mechanical and structural properties of the scaffold can direct cellular activity and further determine the functionality of a tissue-engineered construct.[10-13] In this context, the aim of this study was to investigate the effects of dehydrothermal crosslinking treatment on the mechanical and structural properties of a CG scaffold used for tissue engineering applications . Temperature (105–180°C) and exposure period (24–120 h) of DHT treatment were varied to determine the effects of these parameters on the mechanical properties, crosslinking density and denaturation of CG scaffolds. As expected, increasing the temperature and duration of DHT treatment

resulted in an increase in the mechanical properties. Compressive properties increased up to 2-fold, while tensile properties increased up to 3.8-fold. Crosslink density increased with DHT temperature but not exposure period. Denaturation increased with DHT temperature and exposure period, ranging from 20% to 60% denaturation. Taken together, these results indicate that DHT treatment is a viable technique for altering the mechanical properties of CG scaffolds. Accordingly, DHT treatment can be used to produce CG scaffolds with a range of mechanical properties and denaturation level. This provides both a means to tailor CG scaffolds for specific tissue engineering applications and a 3D *in vitro* model system to study how mechanical properties and denaturation affect cell function.

In this study we investigated the effects of varying DHT parameters on both compressive and tensile properties of CG scaffolds. The effects of DHT parameters on crosslink density and denaturation were also investigated and correlated with the mechanical properties. The detailed analysis of DHT treatment and the resulting effects is a key strength of this study. In contrast, previous studies have predominantly investigated the effects of DHT treatment on collagen solubility or denaturation temperature.[16,20] Further to this, studies are often carried out on collagen fibres or films [16,18-20], which may not be comparable to the crosslinking of a CG co-polymer. Studies that have examined the mechanical properties of DHT-treated collagen have limited their investigation to tensile properties due to the difficulties of testing collagen fibres in compression.[18,19] Studies investigating crosslinking treatments commonly evaluate the *in vitro* cytotoxicity of the method used.[29,30] Whilst the effect of crosslinking on the viability of cells seeded on the

scaffolds is important, DHT treatment does not introduce any potential cytotoxic reagents into the scaffold [31] and the viability of cells seeded on CG scaffolds crosslinked in this manner has been demonstrated extensively in the literature.[5,6,29,32,33]

Through DHT treatment, we produced CG scaffolds with moduli ranging from 0.5–1 kPa in compression and 1.9–7.6 kPa in tension (Fig. 1). These results compare well with previous studies.[18,34,35] After crosslinking CG scaffolds at 105°C for 24 h, Harley *et al.* reported a compressive modulus of 0.2 kPa and a tensile modulus of 2.0 kPa [34], which compare well with our results of 0.5 kPa in compression and 1.9 kPa in tension. In the study by Harley *et al*., the compressive modulus was calculated from a linear fit to the stress-strain curve over 0–10% strain, while we limited evaluation to 2–5% strain. The 0–2% strain regime encompasses the less stiff 'toe' region of the curve, explaining the lower compressive modulus reported by Harley *et al.*

FT-IR analysis supported the theory that DHT treatment forms crosslinks through a condensation reaction between carboxyl and amino groups. During this reaction NH_2 is converted to NH [28], which was monitored using FT-IR. Results show that the crosslink density increased with the temperature of DHT treatment but was not affected by exposure period (Fig. 2 A). However, it may be possible that additional crosslinks are formed by extended DHT treatment. For example, it has also been postulated that a lysino-alanine crosslink may also be formed during DHT treatment.[15,36] This type of crosslink would not be detected using FT-IR analysis.

Results confirm that the extended exposure to high temperatures during DHT treatment alters the structure of the collagen in CG scaffolds. FT-IR analysis demonstrated that the level of denaturation caused by DHT treatment increased with exposure period and temperature (Fig. 2 B). Scaffolds were 25% denatured after 24 h at 105°C, which compares well with a previous report of 27% denaturation for a collagen film after treatment at 110°C.[20] Denaturation increased to 60% after DHT treatment at 180°C for 120 h. Extended treatment at 180°C also produced a decrease in tensile and compressive moduli, which may be indicative of changes in scaffold integrity caused by thermal degradation at this temperature. The temperatures used during DHT treatment break the hydrogen bonds that maintain the triple helical structure of collagen, altering it to a random coiled structure.[37,38] Previous work suggests that denatured collagen matrices show better tissue regeneration than native collagen matrices.[22] Therefore, the increased denaturation at higher temperatures coupled with the enhanced mechanical properties could provide a superior scaffold for tissue engineering. However, denatured collagen can also degrade faster [30], so further investigation is required to determine if the increased denaturation has any effects on cellular activity and scaffold integrity both *in vitro* and *in vivo*.

Previous studies on collagen films have reported a decrease in tensile modulus and UTS with increasing denaturation.[39,40] While we found that the UTS and strain to failure were both were negatively correlated with denaturation (Fig. 4), the tensile modulus of CG scaffolds was positively correlated with denaturation (Fig. 3 B). Therefore, DHT treatment, and possibly denaturation itself, causes the CG scaffold to become more

brittle. However, the unwinding of the collagen triple helix caused by denaturation may allow the formation of additional crosslinks by bringing side chains into alignment. This would increase modulus while increasing brittleness. In contrast to tensile modulus, compressive modulus was only correlated with crosslink density. This suggests that structural changes that occur during DHT treatment, either through denaturation or the formation of crosslinks not detected using FT-IR, restrict fibre sliding but do not affect fibre buckling or bending stiffness.

CONCLUSION

Previous work has shown that mechanical properties must be considered when designing a scaffold for a specific tissue engineering application.[10,13,32,41] This study demonstrates that tensile and compressive moduli of CG scaffolds can be significantly increased through DHT treatment. Both the temperature and duration of DHT treatment were found to have a significant effect on scaffold moduli and denaturation. Results show a correlation between increases in tensile modulus and the level of denaturation. The enhanced mechanical properties of DHT-treated CG scaffolds improves their suitability for use both *in vitro* and *in vivo*. In addition, this work facilitates investigation into the effects of mechanical properties and denaturation on cellular activity in a 3D environment.

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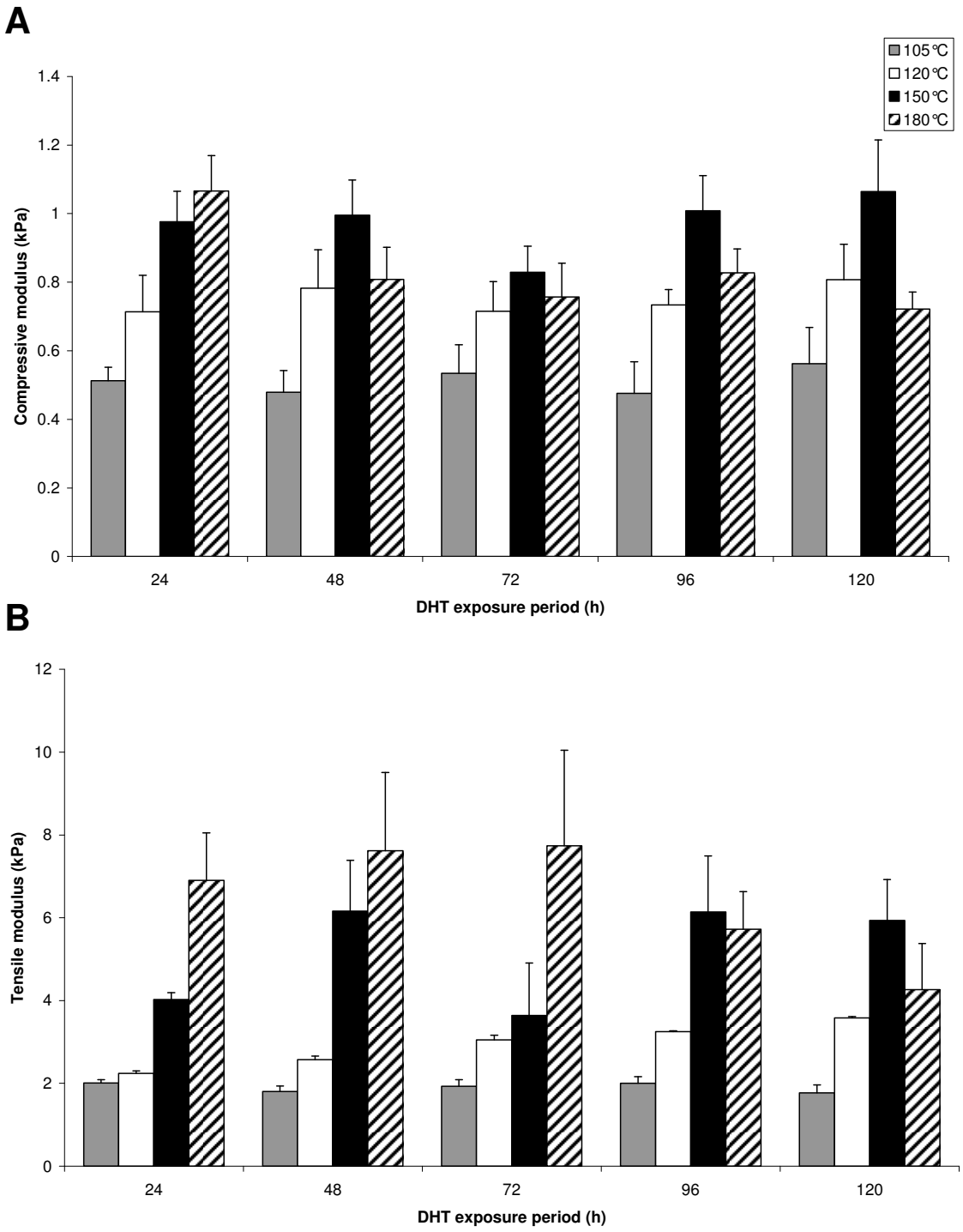

Figure 1. Compressive and tensile moduli of DHT treated CG scaffolds. **A)** Compressive modulus versus DHT exposure period at four different temperatures (n=10 samples per temperature/duration combination). **B)** Tensile modulus versus DHT exposure period at four different temperatures (n=6 samples per temperature/duration combination).

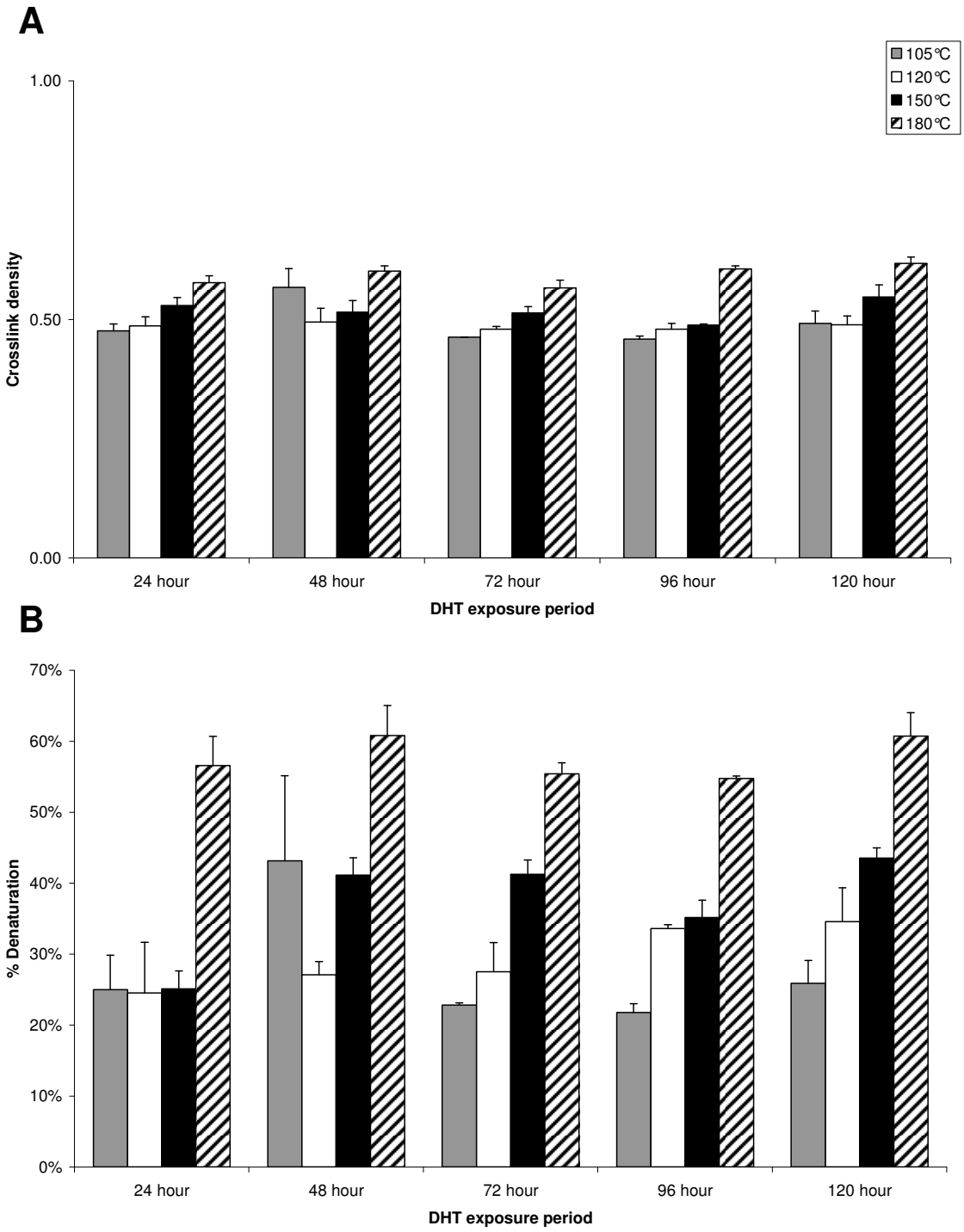

Figure 2. Denaturation and crosslink density after DHT treatment. **A)** Crosslink density versus DHT exposure period at four different temperatures (n=3 samples per temperature/duration combination). **B)** Denaturation versus DHT exposure period at four different temperatures (n=3 samples per temperature/duration combination).

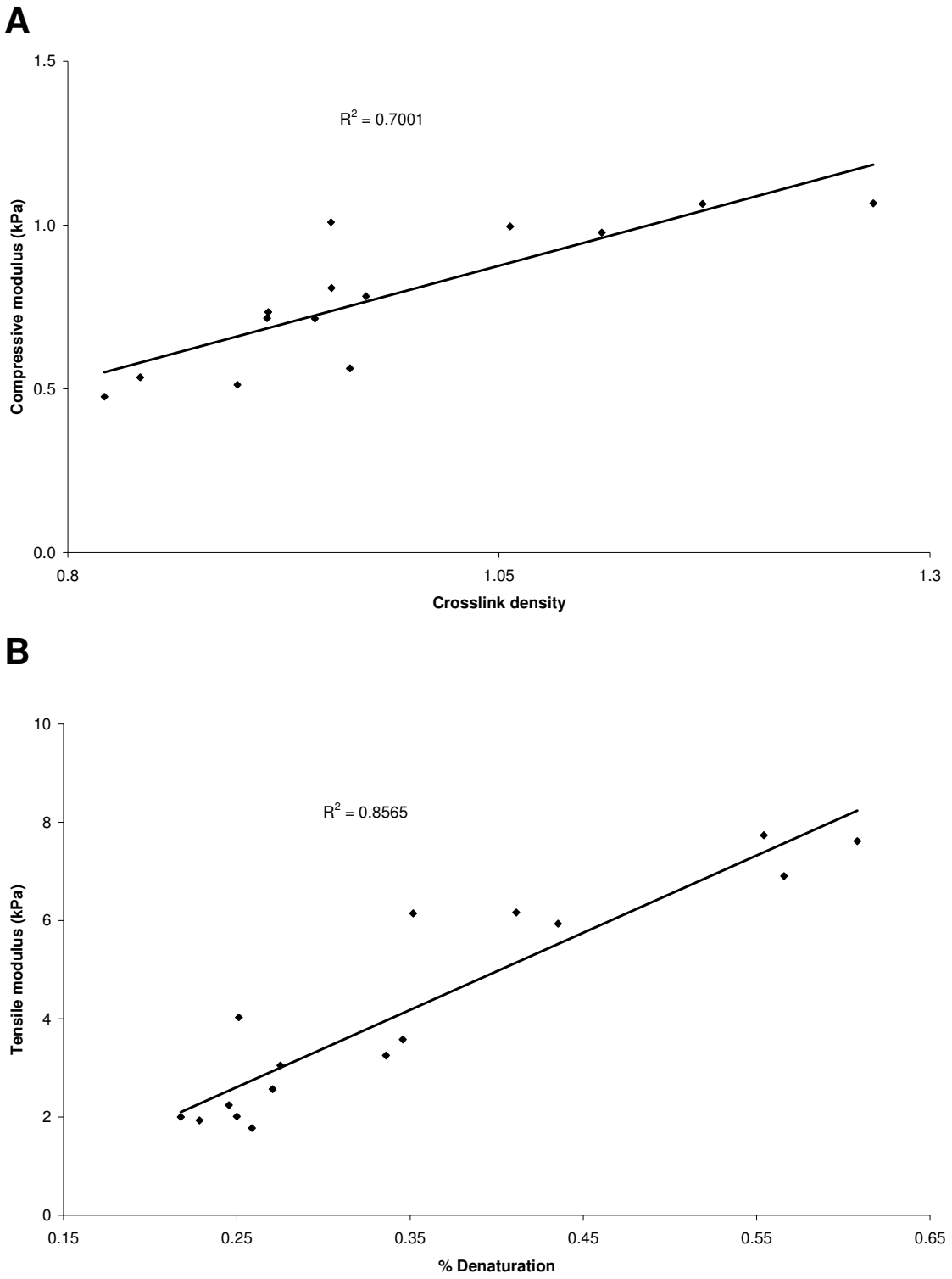

Figure 3. Linear regressions of moduli against crosslink density and denaturation. **A)** Compressive modulus correlated with crosslink density. **B)** Tensile modulus correlated with denaturation.

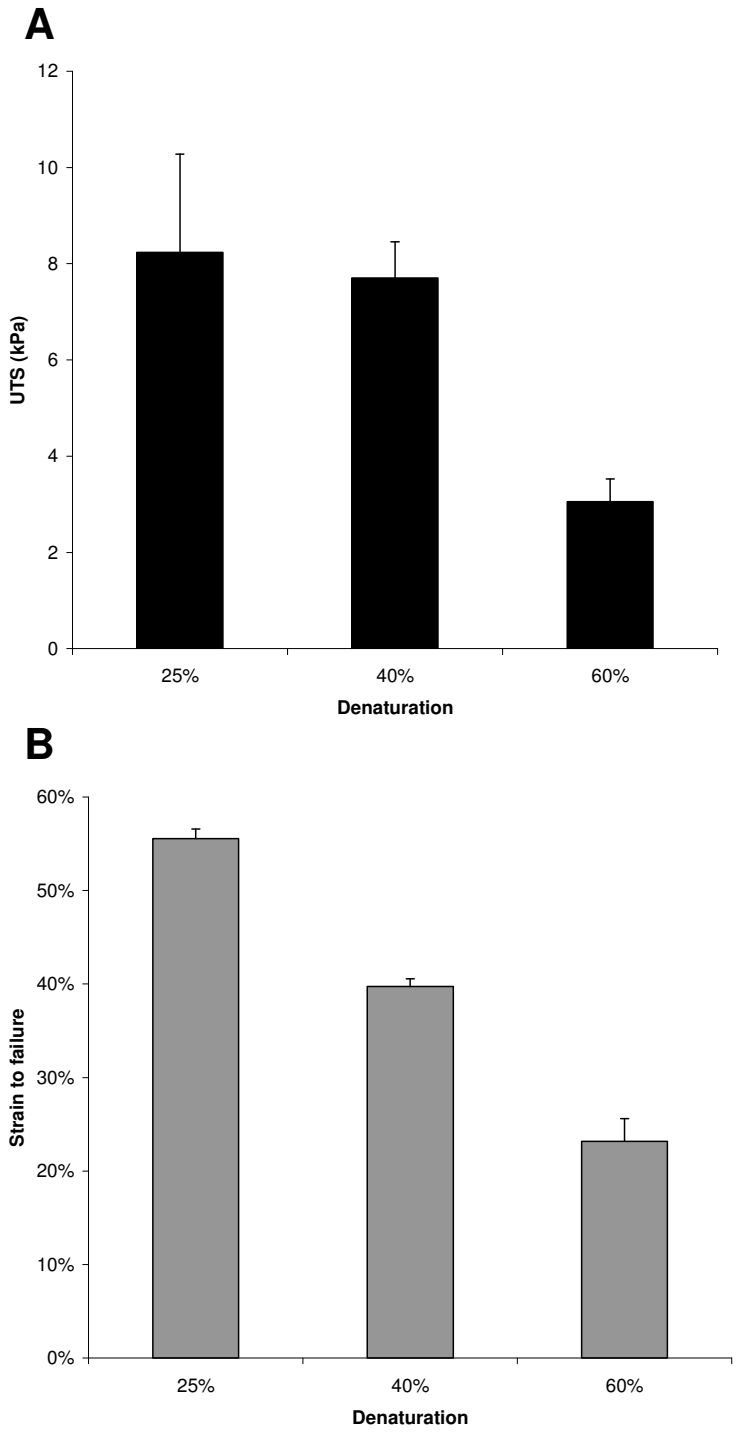

Figure 4. The relationship between tensile properties and denaturation **A)** Ultimate tensile strength versus % denaturation (n=3 per group). **B)**. Strain to failure versus % denaturation (n=3 per group).