Development and use of animal models to advance tendinopathy research

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1. ABSTRACT

Tendon overuse resulting in the development of tendinopathy is a common and significant clinical problem. Until recently, the pathology underlying tendinopathy was thought to be associated with inflammation and was subsequently categorized as 'tendinitis'. However, histopathological studies have indicated the underlying pathology to be one of failed healing or degeneration ('tendinosis'). This clarification and correct labeling of the pathology has substantially altered clinical thinking and management of tendon overuse conditions. It has also stimulated interest in new lines of tendon research. To rapidly understand more about clinical tendinopathy, there is a need for validated animals of the underlying pathology. This paper discusses the use of animal models in tendinopathy research. It discusses the benefits and development of animal models of tendinopathy, highlights outcome measures that may be used in animal tendon research, reviews current animal models of tendinopathy, and discusses methods to enhance outcomes from animal models. With further development of animal models of tendinopathy, new strategies for the prevention and treatment of tendinopathy in humans may be generated.

2. INTRODUCTION

Tendons connect muscle to bone to complete a mechanical series that functions to transmit, store and attenuate forces necessary for motion. In fulfilling these roles, tendons are exposed to repeated tensile loads of varying magnitude. The core tendon structure of collagen arranged in hierarchical levels of increasing complexity is designed to withstand tensile loads, yet it is not uncommon for repeated loading to result in the development of a tendon overuse injury. The most common and significant of these injuries is termed tendinopathy (*tendo-* = tendon, -*pathy* = disease). Tendinopathy refers to a clinical condition characterized by activity-related pain, focal tendon tenderness and intratendinous imaging changes. Its prevalence is as high as 45% in elite athletes depending on the specific physical endeavor and tendon location (1), and can require in excess of six months for recovery of symptoms with first-line conservative management (2).

Despite the clinical significance of tendinopathy, progress in understanding the underlying pathology has only recently progressed. Histopathological studies have shown the pathology to be one of failed healing response,

at times accompanied by progressive tendon degeneration (tendinosis), rather than the traditionally assumed inflammation (tendinitis) (3-5). Given the recent identification of the pathology underlying tendinopathy, knowledge regarding its pathophysiology is incomplete. This has restricted management options, with many current interventions being based on clinical experience and theoretical rationale rather than the manipulation of underlying pathophysiological pathways (6).

Contributing to the current voids in our understanding of tendinopathy is a lack of validated animal models of the underlying pathology (7). Suitable animal models of tendinopathy are required to advance our understanding of the clinical issues of tendinopathy. This paper discusses the development and use of animal models in this field. It discusses the benefits and development of animal models of tendinopathy, highlights potential outcome measures that may be used in animal tendon research, reviews current animal models of tendinopathy, and discusses methods to enhance outcomes from animal models.

3. BENEFITS OF ANIMAL MODELS IN TENDINOPATHY RESEARCH

Tendinopathy research involving animals offers numerous unique benefits over similarly performed research in humans. Principally, animal models enable studies to be performed using in-depth invasive analyses at the organ, tissue and molecular levels. It is possible to assess human tendons at similar levels by collecting biopsy (8) and surgical samples (3, 9-11), or harvesting tissue post-mortem (3, 11). However, all these methods are relatively invasive.

The ability to perform invasive analyses in animal models provides researchers with powerful tools to advance understanding of many aspects of tendinopathy. By elucidating early changes associated with the development of tendinopathy in animal tendons, pathways associated with the initiation and progression of tendon damage may be discovered, potentially leading to the development of novel preventative strategies. These pathways are near impossible to explore in humans, as initial tissue changes associated with tendinopathy most likely precede the onset of symptoms, and it is difficult to justify harvesting asymptomatic tendon tissue from humans.

Animal models also permit more in-depth investigation of changes associated with established tendinopathy, allowing current interventions to be validated, and potential novel targets to be revealed. In terms of the latter, it is typically a requirement that novel compounds undergo pre-clinical evaluation in animal models to establish their efficacy and safety before use in clinical trials. A prerequisite for this in tendinopathy research is the establishment of valid animal models of tendinopathy.

Animal models facilitate the search for novel pathogenic pathways by providing researchers with the flexibility to control variability. For instance, in animal models it is possible to isolate the effects of single genetic or environmental variables while controlling other potential confounders. This is difficult to perform in clinical studies, given the vast differences in genetic and environmental backgrounds among individuals, which results in large variability and the need for large sample groups to achieve sufficient statistical power.

4. DEVELOPMENT AND PATHOLOGY OF HUMAN TENDINOPATHY

For an animal model of tendinopathy to be considered valid, it needs to accurately represent the condition in question as it occurs in humans. As with most animal models, this typically involves introducing known risk factors for the condition as it occurs in humans, and comparing the resultant pathology to that observed in humans.

4.1. Risk factors for tendinopathy in humans

Introducing human pathogenic factors for tendinopathy is a logical approach to producing tendinopathy in animals. Unfortunately, this is difficult, as the mechanisms by which tendinopathy develops in humans are still largely unknown. Conceptually, as with most overuse conditions, the development of tendinopathy likely results from the interplay of two groups of factors, namely extrinsic and intrinsic factors (12). Extrinsic factors refer to factors in the environment or external to an individual that influence the likelihood of developing tendinopathy. Intrinsic factors refer to processes internal to an individual that influence their response to the extrinsic factors.

The initiation of tendinopathy in humans is most commonly blamed on extrinsic factors, with mechanical overload being the single most frequently reported causative factor. For tendinopathy to develop, repeated submaximal loading of a tendon is typically required. This explains its higher prevalence in individuals involved in active endeavors. Although extrinsic factors are the most consistent causative factor for the development of tendinopathy, its development in some individuals (whilst others with equivalent loading are spared) indicates that intrinsic factors must also contribute. Many intrinsic factors have been postulated as contributors, including age, gender, body weight, gene polymorphisms, and anatomical and biomechanical variations (13-16). These risk factors do not independently cause tendinopathy, but their presence potentiates the development of tendinopathy when mechanical overload co-exists.

Exactly how extrinsic and intrinsic risk factors combine to produce the initial tissue damage for the onset of tendinopathy is not known. Tendons are mechanosensitive, and respond and adapt to their mechanical environment (17). However, repeated heavy loading, with or without the presence of one or more intrinsic risk factors, may produce initial pathological

changes in either the extracellular matrix (ECM) or cellular components of a tendon.

The ECM theory suggests that strains below failure levels are capable of generating damage when introduced repetitively. A natural phenomenon associated with repetitive subfailure strain in tendon may thus produce damage (termed microdamage) (18). This damage is of little consequence in most instances, as tendons are capable of intrinsic repair. However, under certain conditions, imbalances can develop between damage production and its repair. The resultant damage accumulation is theorized to be the start of a pathology continuum that results in tendinopathy, and ultimately the development of symptomatic tendinopathy.

An alternative to the ECM theory is that matrix changes are actually preceded and initiated by cellular changes. This cell-based theory stems from findings showing that the cells responsible for tendon maintenance display potentially pathological responses to loading. Tendon is primarily maintained by resident tenocytes, which have the dual function of producing both catabolic and anabolic responses. Both of these functions are modulated by mechanical loading. For instance, in response to fluid-induced shear stress, tenocyte expression of house-keeping, stress response and transport-related transcripts are up-regulated, whereas apoptosis, cell division and signaling-related genes are largely down-regulated (19). These changes are consistent with an anabolic tendon response. However, tenocytes also exhibit a number of changes in response to loading that are consistent with reduced matrix production and enhanced matrix degradation. These include increased apoptosis (20) and synthesis of prostaglandin-E_2 (21, 22). These have been linked to decreased matrix production (23, 24) and increased matrix metalloproteinase expression (25), which is suggestive of matrix degradation. These cellular-derived changes may be involved in the initial development of tendinopathy.

4.2. Pathology of human tendinopathy

To develop animal models of tendinopathy, the models need to be validated against the condition in humans. While the pathology and pathophysiology of tendinopathy in humans is currently poorly understood, two common and established features upon which to validate animal models are histopathological changes and mechanical weakening of the tendon.

Histopathological studies show that tendinopathy in humans (3-5) is characterized histologically by a failed reparative response and an absence of inflammatory cells (3, 5, 26-29). The pathological region is distinct from normal tendon with both matrix and cellular changes. Matrix changes include expansion of the tendinous tissue, loss of the longitudinal alignment of collagen fibers, loss of the clear demarcation between adjacent collagen bundles, and extensive neovascularization (3, 5, 26-28, 30-33). Cellular changes include hypercellularity resulting from atypical fibroblast and endothelial cellular proliferation

(26, 33), and altered morphology of the collagen-producing tenocytes (5, 34).

The histopathological changes associated with tendinopathy may result in physical weakening of the affected tendon. Tendons must be able to withstand large-magnitude tensile loads in their function. When tendinopathy is present, the ability of the affected tendon to withstand large-magnitude tensile loads is compromized, resulting in a higher propensity for mechanical failure (tendon rupture). This was most elegantly demonstrated by Kannus and Jozsa (11) who showed that degenerative pathological changes preexisted in 97% of spontaneously ruptured tendons. Similar findings have been found by other authors, with rotator cuff tears (35), and patellar (36) and Achilles (37-39) tendon ruptures all being associated with underlying tendinopathy.

Animal models should replicate the above histopathological and mechanical features of tendinopathy in humans to be considered valid. Once new findings in humans are derived and established, the animal models need to be re-evaluated and re-validated. For instance, molecular studies performed on human tendon samples have recently demonstrated tendinopathy to be associated with distinct changes in gene expression and matrix turnover (40). Once these changes become more firmly established in human tendon samples, their presence needs to be confirmed in the available animal models to reconfirm the models' validity.

5. DEVELOPMENT OF TENDINOPATHY IN ANIMAL MODELS

To develop tendinopathy in an animal model, researchers commonly reproduce perturbations reflective of the key risk factors for the condition in humans. This is in an attempt to initiate and produce tendon damage consistent with that observed in humans. As per human risk factors, risk factors in animal models can be grouped into extrinsic and intrinsic.

Mechanical overload is the most commonly introduced extrinsic risk factor for the development of tendinopathy in animal models. This is intuitive, considering the proposed ECM theory for the generation of tendinopathy, and that mechanical overload is the most frequently reported causative factor in humans. Mechanical overload of animal tendons is typically introduced using forced treadmill running (41, 42), tendon loading via artificial muscle stimulation (43-46), and direct tendon stretching via an external loading device (47).

Treadmill running is commonly used to induce adaptation in the animal musculoskeletal system. However, it has had variable success in producing overuse tendon pathologies. The predominant reason for this is that studies employing treadmill running have typically used animal species that are habitual runners, such as rodents. Rodents run in excess of 8 km/night in the wild (48), and up to 15 km/day in voluntary wheel running studies (49, 50). This

preference for running facilitates acclimation of rodents to treadmill training. However, it makes it difficult to induce overuse injuries in this species, as their musculoskeletal system is inherently adapted for running. To induce pathological changes, the musculoskeletal system needs to be stressed beyond customary levels. It has proven difficult to force rodents to run at levels in excess of those experienced during voluntary running. For instance, most treadmill studies run animals at speeds less than 20 m/min and for less than 2 hr/day, which equates to a traveled distance of less than 2.5 km/day. Increasing running frequency, duration and/or intensity (increasing belt speed or belt angle from the horizontal) is difficult, as it often coupled with animal resistance and elevated stress (51).

Alternative methods of overloading animal tendons are to use artificial muscle stimulation and direct stretching of a tendon. Both methods are usually involuntary, as they typically involve animal anesthesia, yet they have the advantage of permitting within-animal studies designs wherein overloaded tendons can be compared to contralateral control tendons. Artificial muscle stimulation via electrode stimulation results in tendon loading as tendons function to transmit muscle contractile forces to the skeleton for motion. By coupling muscle stimulation with simultaneous resistance of free segment motion, tendon stress can be elevated. Performing this repetitively may result in tendon degradation and the development of tendinopathy. Similarly, tendinopathy may be developed by directly stretching a tendon. This requires surgery to enable the tendon to be mechanically lengthened via an external device. As tendons are difficult to grip without producing a compressive injury, direct mechanical loading is really only feasible with the patellar tendon where the dual bone attachments of the patellar tendon can be distracted without directly damaging the tendon substance (47).

The introduction of extrinsic factors in isolation has had some success in developing tendinopathy in animal models (42, 46, 47). However, success has been limited to specific situations. For instance, treadmill running successfully produces tendinopathy in the rat supraspinatus tendon (52), but not in the Achilles tendon (41), while artificial muscle stimulation produces rabbit flexor digitorum profundus (FDP) tendinopathy (46), but not Achilles tendinopathy (43, 45). As a result of this variable success, and the fact that the introduction of extrinsic factors is labor intensive, researchers have investigated the potential of intrinsic factors in the development of tendinopathy in animal models.

The introduction of intrinsic factors in animal models typically involves intratendinous injection of chemical compounds, such as collagenase, prostaglandins-E_1 and -E_2, corticosteroids and cytokines (53-59). Introduction of these compounds produces both histological and mechanical changes within the injected tendon. However, their isolated introduction does not appear sufficient to induce the development of a pathology that replicates that observed in human tendons. For instance, the expression of collagenase is elevated in human tissues with tendinopathy, and in animals catalyzes the breakdown of collagen (60). However, intratendinous injection of collagenase results an acute and intense inflammatory reaction (tendinitis) (53, 61, 62), followed by progressive tendon reparation (42, 62). This does not replicate the pathology observed in humans. While it is possible that initial tissue changes associated with tendinopathy in humans include inflammatory pathways, these events are thought to occur well before the onset of symptoms, and are not evident in established tendinopathy. Thus, the use of intrinsic factors in isolation is questionable.

The best approach may to combine these with an extrinsic factor given the apparent dependence of human tendinopathy on mechanical overload. This approach has been shown to result in greater degeneration of the rat supraspinatus (42, 63, 64) and Achilles (61) tendons.

6. OUTCOME MEASURES IN ANIMAL MODELS OF TENDINOPATHY

The use of animal models in tendinopathy research enables the investigation of a wide-range of outcomes measures. It is possible to obtain clinically-translatable *in vivo* outcomes in animal models using clinical imaging techniques (such as ultrasound biomicroscopy and magnetic resonance imaging) which have been modified to provide ultra-high resolution of tendon morphology. However, the real benefit of animal models of tendinopathy derives from the ability to perform a wide range of *ex vivo* outcome measures at all levels, including the organ, tissue, and molecular levels.

The primary *ex vivo* measures of interest in animal models of tendinopathy are the mechanical properties of the tendon. Reduced mechanical properties resulting in an increased likelihood of spontaneous rupture is the ultimate consequence of clinical tendinopathy (11). Mechanical testing with careful consideration to testing set-up can be performed on both large (i.e. horse) (65) and small (i.e. mouse) (66, 67) animal tendons. Key variables of interest include both low- and high-load properties. The former provide information on viscoelastic properties (such as creep and stress-relaxation), while the latter provide information on both tendon structural (such as ultimate force and stiffness) and material (such as ultimate stress and strain, and elastic modulus) properties.

Mechanical testing of animal tendons provides valuable information on the effects of tendinopathy on tendon function. However, from a disease etiology and intervention perspective, animal models are invaluable in light of the mechanistic information they can provide at the tissue and molecular levels. At the tissue level, there are numerous useful techniques that can be applied in animal models that provide information on tissue structure and composition. For instance, standard histological techniques can provide information regarding collagen fiber arrangement, cellular composition and vascularity, while

advanced histological techniques such as immunohistochemistry and *in situ* hybridization can be useful for the localization of specific proteins, and DNA and RNA sequences. Tissue-level properties of animal tendons can also be explored using high-powered electron microscopy techniques. These allow the investigation of collagen fibril arrangement and morphology, factors that influence tendon mechanics.

Potential mechanisms for organ and tissue level changes associated with tendinopathy in animal models can be explored in depth using powerful molecular techniques. Tissue composition can be determined using chromatography techniques, with important features being ECM (collagens), proteoglycan (including decorin, biglycan, fibromodulin) and glycoprotein (including elastin, fibrillin, tenascin-C) content. Meanwhile, pathways responsible for differences in tissue composition can be elucidated using a combination of microarray analyses, polymerase chain reactions (PCR), Western blots, electrophoresis and mass spectrometry. Microarray analysis allows the simultaneous measurement of the expression levels for tens of thousands of genes permitting the molecular aspects of tendinopathy pathogenesis and intervention to be modeled. As microarray analysis only provides information on relative expression levels, it needs to be coupled with a quantification method such as PCR. Real-time PCR amplifies specific reverse transcribed transcripts, allowing for their detection and quantification. Since transcript levels may not be directly proportional to protein production, PCR subsequently needs to be coupled with a method for protein quantification, such as western blots, electrophoresis or mass spectrometry. Further details on these potential outcome measures in animal tendon studies have been detailed elsewhere (68).

7. CURRENT ANIMAL MODELS OF TENDINOPATHY

Using the aforementioned approaches and techniques, researchers have generated and assessed tendinopathy in a number of animal models. These have included rat models of supraspinatus and patellar tendinopathy, and a rabbit model of FDP tendinopathy. The models of naturally occurring tendinopathy in horses and dogs will not be reviewed here as their large size and cost negates their ability to be widely used and practical animal models.

7.1. Rat model of supraspinatus tendinopathy

The rat model of supraspinatus tendinopathy, first described by Soslowsky and colleagues (52), is the most established animal model for human tendinopathy. The model involves running rats on a treadmill at 17 m/min for 1 hr/day and 5 days/wk, which equates to a daily running distance of 1 km. This distance appears insufficient to overload the rodent musculoskeletal system in isolation. However, it produces tendinopathic features when coupled with a 10° decline of the treadmill. Decline running results in a relative shift of load from the hindlimbs to forelimbs during quadruped gait. This theoretically facilitates narrowing of the subacromial space, resulting in impingement of the supraspinatus tendon. When this impingement is coupled with the approximately 7500 strides per day taken by rats during a treadmill session, it is sufficient to cause tendinopathy of the supraspinatus tendon.

Tendinopathy induced in the rat supraspinatus tendon has similar features to those observed in humans. Histologically, decline running in rats produces supraspinatus tendon hypercellularity and irregular collagen fibril arrangement (52, 63, 64). These tissue changes are coupled with mechanical decay, with supraspinatus tendons from rats who performed decline running having reduced structural (ultimate force and stiffness) and material (ultimate stress and elastic modulus) properties (52, 63, 64). These changes are evident as early as 4 wks following the initiation of running (52, 63, 64), and are exacerbated by combined introduction of an intrinsic risk factor such as collagenase injection or anatomical narrowing of the subacromial space (42, 63, 64).

As the histological and mechanical changes induced in rat model of supraspinatus tendinopathy are representative of those observed in humans, the model has gained acceptance by other researchers (69, 70). The utility and popularity of the model is also enhanced by its use of an extrinsic factor as the principal pathology inducing factor, which facilitates the translatability of findings to clinical tendinopathy.

7.2. Rat model of patellar tendinopathy

A novel rat model of patellar tendinopathy has recently been described by Flatow and colleagues (47, 71). The model utilizes the ECM theory for the generation of tendinopathy, and involves direct loading of the patellar tendon. Skin incisions are made over the knee joint, and the patella and tibia gripped and distracted to apply repetitive subfailure loads to the patellar tendon. As controlled loading is directly applied to the tendon on an anesthetized animal, the model is able to produce consistent levels of tendon damage independent of other factors (such as animal compliance and muscle fatigue). Potential limitations of the model include the need for surgical exposure of the tendon for loading, and the single loading bout used to induce fatigue damage. A single bout of cyclic loading is introduced until a prescribed loss of secant stiffness (72). While this has been shown to induce histological and mechanical changes consistent with those observed in humans (47, 71, 73), questions remain regarding whether a single bout of loading appropriately represents human tendinopathy.

7.3. Rabbit model of flexor digitorum profundus tendinopathy

A rabbit model of FDP tendinopathy at the medial elbow epicondyle has been described by Rempel and colleagues (46). Following anesthesia, the FDP muscle of one forelimb was electrically stimulated to contract repetitively for 2 h/day, 3 d/wk until 80 cumulative hours

of loading. Finger motion was resisted to facilitate loading of the FDP tendon and permit feedback about loading levels. By the end of the loading regime, cyclically loaded tendons had greater indexes of microstructural damage compared to contralateral tendons, including increased microtear area as a percent of tendon area, microtear density, and mean microtear size (46). Subsequently, the investigators found that tendon cells increased their production of growth factors (vascular endothelial growth factor and its receptor, and connective tissue growth factor) (74). These changes are consistent with attempted healing and may be important in tendinopathy pathogenesis.

8. LIMITATIONS OF ANIMAL MODELS IN TENDINOPATHY RESEARCH

Animal models in research offer numerous benefits. However, their use is not without limitation or controversy. Questions have recently been raised regarding the clinical translatability of data obtained from animal models (75, 76). For instance, in a systematic review of 76 highly cited animal studies, Hackam and Redelmeier (75) found that just over one-third translated at the level of human randomized trials. Similarly, Perel et al (76) found discordance between the findings of animal and human studies for the six conditions and interventions they systematically reviewed.

The discordance in results between animal and human studies for other conditions suggests that animal models may not be useful in advancing tendinopathy research. Reasons for this may include the inadequacy of animal models to adequately mimic the human pathophysiology, and, more simply, the fact that animals typically have a quadruped gait that displays substantial biomechanical differences to the bipedal gait of humans. However, based on the infancy of tendinopathy research and shear lack of studies in both the animal and clinical domains, it is premature and not currently possible to systematically assess the clinical predictability of animal models of tendinopathy.

9. ENHANCING OUTCOMES FROM ANIMAL STUDIES

While it is not currently possible to determine the clinical predictability of animal models of tendinopathy, there are numerous methodological approaches that should be considered to facilitate model predictability. An overriding reason why the findings of animal studies for other conditions are often not replicated in the clinical setting is the presence of significant methodological biases in the design and execution of the animal studies (75, 76). Biases include the lack of animal randomization, non-implementation of concealed allocation, and unblinded assessment of outcome measures. These biases are associated with a greater likelihood that an animal study will find a significant outcome. For instance, a review of 290 animal experiments found that studies conducted

without randomization and blinding are five times more likely to report a positive treatment effect compared to studies that use these more rigorous methods (77).

A further methodological reason why animal studies may not predict clinical results is the frequent statistical underpowering of animal studies. Animal studies typically use limited sample sizes that have not been appropriately powered *a priori*. For instance, Perel et al (76) found only eight of 113 animal studies on thrombolysis for stroke reported a sample size calculation, a fundamental step in ensuring an appropriately powered precise estimate of effect. The lack of *a priori* sample size determinations in animal studies increases the chances of committing a type-II statistical error wherein an effect is not found despite it actually existing. However, it also increases the rate of publication bias in animal research. Animal studies with small sample sizes are more likely to report higher estimates of effect than studies with larger numbers (78, 79) and, therefore, are more likely to be published.

To responsibly advance tendinopathy research using animal models in the absence of clear evidence that the models are predictive of the clinical scenario, studies need to be appropriately designed and executed. There are currently no uniform reporting requirements for animal-based studies. Until these are developed, researchers using animal models to study and advance knowledge of tendinopathy are encouraged to perform *a priori* sample size calculations based on realistic and clinically-relevant effect sizes, randomize animals to experimental groups, and ensure that investigators performing outcome measures are blind to group allocation.

10. CONCLUSIONS AND PERSPECTIVES

Animal models of tendinopathy represent efficient and effective means of advancing our understanding of human tendinopathy. By introducing known risk factors for tendinopathy in humans, it is possible to develop tendon changes in animal models that appear consistent with the human condition. Preliminary animal models have achieved this. However, there is a need for much further research into these and other models. Also, there is a need for additional validated animal models since no single model will answer all research questions. Human tendinopathy occurs at multiple sites, with potentially differing pathologies, and probably as a result of multiple known and unknown risk factors. Animal models for each human scenario are desirable. For these models to be influential they need to be conducted with careful consideration to experimental design. In particular, researchers should perform a priori sample size calculations based on realistic and clinically-relevant effect sizes, randomize animals to experimental groups, and ensure that investigators performing outcome measures are blind to group allocation. Hopefully, using these approaches and with further development, animal models of tendinopathy will lead to the generation of novel preventive and management strategies for tendinopathy in humans.

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