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The tree shrews: adjuncts and alternatives to primates as models for biomedical research

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Abstract: The tree shrews are non-rodent, primate-like, small animals. There is increasing interest in using them to establish animal models for medical and biological research. This review focuses on the use of the tree shrews in *in vivo* studies on viral hepatitis, hepatocellular carcinoma (HCC), myopia, and psychosocial stress. Because of the susceptibility of the tree shrews (*Tupaia belangeri*) and their hepatocytes to infection with human hepatitis B virus (HBV) *in vivo* and *in vitro*, these animals have been used to establish human hepatitis virus-induced hepatitis and human HBV- and aflatoxin B1-associated HCC models. As these animals are phylogenetically close to primates in evolution and have a well-developed visual system and color vision in some species, they have been utilized to establish myopia models. Because dramatic behavioral, physiological, and neuroendocrine changes in subordinate male tree shrews are similar to those observed in depressed human patients, the tree shrews have been successfully employed to experimentally study psychosocial stress. However, the tree shrews holds significant promise as research models and great use could be made of these animals in biomedical research.

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Introduction

The tree shrews (family Tupaiidae) are non-rodent, primate-like animals and are classified into the order Scandentia [49]. They are subdivided into two subfamilies: the diurnal subfamily Tupaiinae with five genera (*Tupaia, Anathana, Dendrogale, Lyonogale, Urogale*) and the nocturnal subfamily *Ptilocercinae* with a single genus, pen-tailed tree shrew *Ptilocercus*. The geographical distribution of the tree shrews extends from India to the Philippines, and from southern China to Java, Borneo, Sumatra and Bali. Natural habitats of these animals are tropical forests and plantation area [18].

Infection with hepatitis viruses, such as hepatitis B virus (HBV) and hepatitis C virus (HCV) is a major human health problem worldwide and associated with a wide spectrum of clinical presentations. Chronic HBV carriers develop chronic hepatitis that often progresses to cirrhosis [1]. Hepatitis virus-associated hepatocellular carcinoma (HCC) is also a major cause of mortality in sub-Sahara Africa, South-east Asia, China, and the Far East and increasingly common in the West [7]. Experimental research in human HBV-induced HCC has been hindered by the paucity of animal models which may be infected by human HBV and subsequently develop HCC. In this respect, the discovery that tree shrews can fulfill the requirements of such an animal model is extremely important.

Myopia has reached epidemic proportions in Asia. The development of myopia may be associated with environmental and genetic factors as well as gene–environment interaction [33]. The tree shrews are phylogenetically close to primates and have especially well-developed eye structures [18]. This has generated recent interest in the use of this model in experimental research in ophthalmology science.

Psychosocial stress is a common phenomenon and involved in a variety of pathophysiological events, such as cardiovascular diseases [21]. As dramatic behavioral, physiological, and neuroendocrine changes in subordinate male tree shrews are similar to those observed in depressed human patients, these animals have been successfully

employed to study psychosocial stress under experimental conditions [16].

In this review, we summarize the known data on the use of animal models established in the tree shrews in biomedical research. In particular, we review how the animal models for viral hepatitis, HCC, myopia, and psychosocial stress are established and their specific applications. We further postulate other possible applications of this animal model in biomedical research.

Virus hepatitis model

Human HBV is a major health problem worldwide and is associated with a wide spectrum of clinical presentations. The asymptomatic HBV carrier may develop cirrhosis, portal hypertension, liver insufficiency and HCC [2]. However, current therapy of chronic HBV infection and its complications are still unsatisfactory. A major problem is the lack of a feasible animal model of human HBV infection. All hepadnaviruses have very narrow host ranges. Efficient infection by human HBV is well-documented for only humans and chimpanzees [42]. Moreover, chimpanzees are restricted in their use as experimental animals in hepatitis research because of their scarcity and the need for their preservation.

Animal models for the related hepadnaviruses of woodchucks, the ground squirrels, and ducks are useful for the research of HBV infection. However, although woodchuck hepatitis virus (WHV) [48], ground squirrel hepatitis virus (GSHV) [28], duck hepatitis B virus (DHBV) [5] belong to the hepadnavirus family, they are different from human HBV and fairly species-specific, infecting only the original host and a limited number of related species. HBV transgenic mice may have chronic hepatitis and liver cancer [24], but this animal model is established by the introduction of HBV DNA into the mouse germ line, which is different from the situation of infection with the intact HBV (Dane) particles.

The tree shrews are close to primates in evolution and unusual in that they are successfully used to establish a human HBV-induced hepatitis model. Primary hepatocytes from the tree shrew *Tupaia belangeri* can be reproducibly infected with human HBV [51]. The *in vitro* infection results in viral DNA and RNA synthesis in the hepatocytes and secreting hepatitis B surface antigen (HBsAg) and hepatitis B envelop antigen (HBeAg) into culture medium. The tree shrews can be also infected with human HBV *in vivo*, resulting in viral DNA replication and gene expression in the livers. Consistent with acute, self-limited hepatitis B in humans, HBsAg is rapidly cleared from serum in the tree shrews, followed by seroconversion to anti-HBe and anti-HBs. The experimental infection rate is 55.2%. Successive infections have been passed through five generations among the tree shrews inoculated with HBV-positive sera from the infected animals, the rate of average infection being 94.0%. The experimental infection of the tree shrews with HBV may be prevented by immunization with hepatitis B vaccine, the protection rate being 88.9% [53]. Human serum interferes with HBV binding to the hepatocytes, thus limiting the maximum multiplicity of infection [25]. Purification of HBV by gradient sedimentation greatly enhances virus binding and infectivity.

The efficiency of infection depends on interactions of the virus with some factors on the surface and within the host hepatocytes. To bypass restrictions during the initial entry phase, recombinant replication-defective adenovirus vectors – either with or without a green fluorescent protein marker gene – can be used to deliver complete HBV genomes into primary hepatocytes from the tree shrews [43]. The human hepatoma cell lines, HepG2 and Huh7, are efficiently transduced by the vectors and produce all HBV gene products required to generate the secretory antigens, such as HBsAg and HBeAg, replication-competent nucleocapsids, and enveloped virions. Covalently closed circular HBV DNA (cccDNA) is also formed. Primary hepatocytes from the tree shrews therefore support all steps of HBV replication following deposition of the genome in the nucleus, including the intracellular amplification cycle. These data provide a rational basis for *in vivo* experiments aimed at developing the tree shrews into a useful experimental animal system for HBV infection.

The tree shrews are not only utilized to study human HBV infection but also used to study HCV infection. The tree shrews can be experimentally infected by human HCV. After whole-body irradiation, the efficiency of infection in the tree shrews may be increased. These animals may serve as an *in vivo* system for culturing human HCV and addressing pathophysiological and therapeutic issues of HCV infection [52]. Currently, an animal model for HCV-associated HCC has not been established in the tree shrews yet.

Hepatocellular carcinoma model

Hepatocellular carcinoma is a major cause of mortality diseases worldwide and accounts for approximately 6% of all human cancers. The rate of annual incidence for HCC is between 350,000

and 400,000 cases in the world. The highest incidence is found in sub-Sahara Africa, Southeast Asia, China, and the Far East [7]. The development of HCC is considered to be a multistep process involving multiple factors. In most high-risk areas, HBV and chemical carcinogens such as aflatoxin B1 (AFB1) are known to play a key role during hepatocarcinogenesis [44]. A study on the hepatocarcinogenic effects of HBV with or without exposure to AFB1 carried out on the tree shrew *T. belangeri* successfully confirmed the complementary roles of these two carcinogenic agents and the value of the tree shrews in such studies. The incidence of HCC was found to be significantly higher in the animals both infected with HBV and exposed to AFB1 (52.9%) than in those solely infected with HBV (11.1%) or exposed to AFB1 (12.5%). No pre-cancerous lesion could be observed in the control group. Both HBV DNA and HBV DNA-encoded proteins were detected in the cancer cells and/or the surrounding hepatocytes. Integration of HBV DNA into the host liver genome was found during hepatocarcinogenesis among the animals infected with HBV. These results suggest that exposure to HBV and AFB1 may play a synergistic role in the pathogenesis of HCC, and support the viewpoint of an etiological relationship between HBV and HCC [54].

The tree shrew HCC model has been used to study the expression and mutations of some genes in HBV- and AF B1-associated hepatocarcinogenesis. The levels of expression of insulin-like growth factor II (IGF-II), p21, and HBV X antigen are significantly higher in the tree shrews infected by HBV and exposed to AFB1 than in the tree shrews solely infected by HBV or exposed to AFB1. The incidence of HCC in these animals is related to the overexpression of these genes [47]. These findings suggest a synergistic effect of HBV and AFB1 in the activation of these genes in the tree shrews. Mutations in the p53 tumor suppressor gene were also found in human HBV- and AFB1-associated HCC in the tree shrews [39]. Point mutations were observed in three of four tree shrew HCC tissues at the p53 gene (C $\rightarrow$ T at codon 13, proline to serine; T $\rightarrow$ C at codon 275, cysteine to arginine; and A $\rightarrow$ G at 78, no amino acid change). Point mutation at codon 275 which is on the DNA-binding domain of p53 gene may be a cause of gain-of-function during hepatocarcinogenesis.

The tree shrews were also useful for evaluating experimental chemo-prevention strategies relevant to high-risk human populations as it mirrors the human epidemiology of liver cancer [26]. To begin developing the model for chemoprevention study, two groups of the tree shrews were fed with AFB1 for 4 weeks. One week before AFB1 administration, one group also received oltipraz for 5 weeks. At weekly intervals, 1 ml of blood and a 24-h urine sample were obtained from each animal. It was found that aflatoxin-albumin adducts increased rapidly in 2 weeks and diminished after cessation of AFB1 exposure. Oltipraz significantly attenuated the overall burden of aflatoxin-albumin adducts throughout the exposure period with a median reduction of 80%. In a single cross-sectional analysis at the end of AFB1 dosing, oltipraz treatment decreased urinary aflatoxin-N(7)-guanine by 93%. Collectively, these results indicate that oltipraz reduces AFB1 risk biomarkers in the tree shrews in a manner similar to that observed in rodents and humans, and establishes a rationale to evaluate cancer chemoprevention by oltipraz in the tree shrews infected with human HBV and exposed to AFB1.

Myopia model

The induction, treatment and prevention of myopia in animals can provide us with important data on the pathogenesis of myopia in humans. Early experimental work was carried out using monkeys as experimental subjects. However, in view of the high cost and the difficulty of obtaining experimental monkeys, myopia has also been induced in different animals, such as the domestic cats, the tree shrews, marmosets, the guinea-pigs and the domestic chicken [6]. Among these animals, the tree shrews are close to primates in evolution and have a well-developed visual system and color vision in some species, such as the tree shrew *T. belangeri* [22, 40]. The tree shrews thus have distinct advantages in myopia research.

Myopia can be induced in the tree shrews by treatment with agents that block collagen cross-linking [β-aminoproprionitrile (β-APN) or D-penicillamine (DPA)] and monocular deprivation (MD) of form vision by eyelid closure [31]. In this model, the interaction of collagen cross-linking with a retinally derived signal regulates the elongation of the eye in myopia. This myopia model has been successfully used to study the changes of sclera during the development and recovery of myopia, such as physiological components, scleral creep, and scleral dry weight.

In the sclera of induced myopia in the tree shrews, the levels of sulfated glycosaminoglycans (GAGs) are significantly reduced relative to DNA and hydroxyproline in the total sclera, implying that proteoglycans or glycosylated or sulfated proteoglycans are reduced in the deprived sclera. The hydroxyproline level is significantly reduced

only at the posterior pole [34]. In recovering eyes, the levels of GAG and DNA synthesis are significantly increased [19, 30]. The decrease of collagen mRNA and the increase of active matrix metalloproteinase 2 (MMP-2) and MMP-2 mRNA can be seen in both the equatorial and posterior sclera of myopic eyes, whereas, active MMP-2 and MMP-2 mRNA are significantly decreased during the recovery of myopia [20, 46]. These results demonstrate that form-deprivation myopia and recovery from myopia alter scleral catabolism and support the theory that changes in eye size during mammalian refractive development are the result of active tissue remodeling rather than passive scleral stretching alone.

The tree shrews have been used to investigate the role of scleral creep in the axial elongation of the tree shrew eyes with induced myopia [41]. Posterior and equatorial scleral samples from myopic eyes have significantly greater creep extensions than those from contralateral control and normal eyes. Among individual tree shrews the difference in creep rate between the sample from the myopic eye and that from the control eye correlates with vitreous chamber elongation and development of myopia in the deprived eye. This result supports the hypothesis that induced changes in the axial length of the mammalian eye are mediated by the changes in the creep properties of the sclera.

The tree shrews have been also utilized to study optical correction of myopia. When myopic eyes in young tree shrews are accurately corrected with a negative lens or have a zero-powered lens placed in front of it, the corrective lenses can prevent recovery from the induced myopia, whereas those with the zero-powered lenses display near full recovery from the induced myopia. Significant reductions in scleral dry weight and glycosaminoglycan synthesis can be also observed in the posterior sclera of the animals wearing corrective lenses, while, the animals wearing zero-powered lenses display elevated levels of lycosaminoglycan synthesis [29]. These results suggest that accurate correction of axial myopia prevents the refractive, biometric and scleral metabolic responses that are normally observed in the tree shrew eyes recovering from induced myopia. These findings support the hypothesis that the recovery of myopia is driven by an active emmetropization response dependent on the clarity of image falling on the retina and not by a mechanism that is sensitive to abnormal eye shape.

When a young tree shrew wears a monocular minus (concave) lens, the vitreous chamber elongates over a period of days, shifting the retinal location to compensate for the altered focal plane. The tree shrew eyes can tolerate altered monocular visual stimulation produced by a minus lens worn for 12 hours of a 14-hours light cycle without developing an induced myopia. However, when the lens is worn for more than 12 hours of 14 hours each day, compensation appears to increase linearly with decreased lens-off time. These results suggest that, if the eyes of human children respond similarly to defocus from near work or other sources, it would seem that the defocus must be present almost all the time to induce myopia. If defocus contributes to human myopia through a compensation mechanism, then an increase in the amount of time that focused images are present should reduce myopic progression [45].

Psychosocial stress model

A variety of evidence indicates an association between life stresses and the pathogenesis of mental illnesses such as depression and anxiety disorders. As simulations within which aspects of depression are investigated in animal models, stresses mostly involve noxious stimuli or perturbations of the physical environment, such as electric foot shock, tail pinch, water and food deprivation, cold exposure, forced swimming, physical restraint, or soiled cages [21]. In recent years, evidence has shown that male tree shrews (*T. belangeri*) represent a suitable model to study the neurobehavioral consequences of chronic psychosocial stress [16]. When two male tree shrews live in the same cage, the subordinate tree shrew shows dramatic behavioral, physiological, and neuroendocrine changes which are similar to those observed in depressed human patients [14].

A key stress response is the activation of the hypothalamus–pituitary–adrenocortical (HPA) axis, resulting in the urinary excretion of cortisol and norepinephrine and the increase of plasma levels of glucocorticoids that have effects on mood and behavior by affecting neurochemical transmission and immune, cardiovascular, and neuroendocrine systems [3, 21]. Corticosterone is the major corticosteroid hormone in the peripheral plasma. Levels of corticosterone and cortisol are markedly elevated in the tree shrews with either restraint stress applied at various times during the day or ACTH administration to both saline- and dexamethasone-pretreated animals but the ratio corticosterone:cortisol is decreased as cortisol is increased more than corticosterone, demonstrating that the adrenal cortex of the tree shrews normally produces corticosteroids through two pathways and the secretion of both corticosterone and cortisol are responsive to acute ACTH stimulation

and feedback inhibition by dexamethasone [4]. Salivary cortisol measurement used in humans for adrenal glucocorticoid hormone analysis can be also used in the tree shrews [37].

Many of behavioral and neuroendocrine reactions are related to those produced by centrally administered corticotropin-releasing hormone (CRH) in laboratory animals and comparable with the symptoms observed in depressed psychiatric patients [38]. Chronic psychosocial conflict in male tree shrews to determine whether long-lasting psychosocial stress would affect CRH binding sites in the brain, the pituitary, and the adrenal gland [15]. Chronic stress significantly reduces the number of CRH binding sites in the anterior lobe of the pituitary, the dentate gyrus, the CA1–CA3 areas of the hippocampus, and both the stratum griseum superficiale and the stratum opticum of the superior colliculus. A significant stress-induced enhancement of CRH binding sites occurs in the frontal cortex, cingulate cortex, claustrocortex, the central and the lateral nucleus of the amygdala, and the choroid plexus. The different response patterns of the central CRH binding sites reflect distinct neuroendocrine processes which are presumed to coordinate behavioral, autonomic, endocrine, and immune responses to the long-lasting psychosocial conflict.

Behavioral changes induced by chronic psychosocial stress in male tree shrews may be related to alterations in the central nervous adrenoceptor systems. In the noradrenergic centers of the brain, α 2-adrenoceptors function as autoreceptors regulating norepinephrine release. Chronic stress downregulates these receptors in several brain regions [10, 13]. During the stress, the activity of the HPA axis is increased leading to high concentrations of plasma glucocorticoids. A short-term treatment with cortisol downregulates α 2-adrenoceptors in several brain regions, whereas, a long-term oral treatment induces regional receptor upregulation, implying that (1) glucocorticoids regulate α 2-adrenoceptors in the brain; (2) the duration and/or the route of cortisol application determines the results; and (3) chronic stress effects are not only the result of the long-term glucocorticoid exposure, but also because of other elements of the stress response [12]. Chronic stress in the tree shrews decreases the expression of the α (2A)-adrenergic autoreceptor in the locus coeruleus and the α (2A)-heteroreceptors in glutamatergic neurons [32]. In addition, chronic psychosocial stress also leads to time-dependant changes in the central nervous β-adrenoceptors system. The high regional variability in stress-induced β-adrenoceptor regulation is supposed to be the result of the complex mechanisms of intracellular β-adrenoceptor sequestration, which includes down-regulation and/or reinsertion of receptors into the plasma membrane. These mechanisms may be important components of the regulatory apparatus which enables the individual to adapt to situations of recurrent stressful experiences by balancing the central nervous adrenoceptor number [8, 9].

The behavior of subordinate male tree shrews is characterized by a reduction in scent marking, self-grooming and overall locomotor activity. This subordination behavior has been proposed to relate to the down-regulation of 5HT1A-receptors occurring in distinct brain regions of the animals [11]. As chronic stress in subordinate male tree shrews was also found to decrease androgen levels, behavior and 5HT1A-receptor expression were observed after testosterone replacement. Although, in subordinate male tree shrews, cortisol levels remain high during the testosterone treatment, 5HT1A-receptors in the hippocampal formation and the occipital cortex are renormalized to control levels by the androgen, but 5HT1A-receptors in the ventromedial thalamic nucleus do not return to baseline levels. Both scent marking and self-grooming behavior are renormalized by testosterone, but overall locomotor activity does not return to baseline levels. These data indicate that a balance between glucocorticoids and androgens is necessary to maintain 'normal' numbers of the monoamine receptors. The fact that 5HT1A-receptors and certain behaviors can be renormalized by the androgen supports the view that 5HT1A-receptors are involved in the regulation of stress behavior. However, the fact that overall locomotor activity is not returned to baseline indicates that different types of behavior are distinctly regulated [17].

Chronic stress in male tree shrews that is accompanied by constantly elevated levels of glucocorticoids leads to structural changes in hippocampal neurons. Dendritic atrophy of hippocampal pyramidal neurons and impairment of neurogenesis in the dentate gyrus can be demonstrated in chronically stressed tree shrews, a loss of hippocampal neurons does not occur in these animals [23, 27, 50].

The tree shrews are first used to evaluate the consequences of sequential stress exposure on memory performance in animals. Chronic stress differentially affects hippocampus-mediated and hippocampus-independent memory processes in the tree shrews. Hippocampus-mediated memory is persistently impaired during either stress or recovery periods, while hippocampus-independent memory processes remain unimpaired throughout the study. This persistent impairment seems

not to be exclusively triggered by glucocorticoids because urinary free cortisol concentration returns to normal during recovery period [35]. In subordinate male tree shrews, psychosocial conflict causes elevated cortisol levels during the stress phases. Despite normalized cortisol levels, significant memory deficits in experimental animals can be observed even 10 weeks after the last stressful experience. However, the negative correlation between the level of adrenal steroid hormones and memory performance does not account for the long-lasting effects of psychosocial stress in the tree shrews [36].

The tree shrews have been used to evaluate the effects of antidepressant drugs on chronic psychosocial stress. The tricyclic antidepressant clomipramine has a time-dependent restorative influence on marking and grooming behavior, locomotor activity, and risk assessment, as well as on urinary cortisol and norepinephrine excretion. It appears that the clomipramine treatment counteracts the behavioral and endocrine effects of chronic psychosocial stress in the tree shrews, and the time course of recovery corresponds closely to that observed when treating depressed patients in the clinic [14].

Other species of the tree shrews as biomedical models?

Currently, the tree shrew *T. belangeri* as animal models has been used to carry out biomedical research. This animal is a native of Yunnan, China, but related species are found all over South-east Asia. These related species may be expected to exhibit some of the characteristics of the *T. belangeri* that has made the tree shrews such good biomedical models. In view of the importance and potential applications of these models, the research on the feasibility of related species as biomedical models should be encouraged.

Conclusion

The tree shrews are phylogenetically close to primates and useful small animal models. Viral hepatitis, HCC, myopia and psychosocial stress are major problems in human beings. The models of these diseases established in the tree shrews should be expected to find increasingly applications for the *in vivo* study of these diseases. They are particularly useful for the study of mechanism involved in the pathogenesis of these diseases. The role of various genes during the development of these diseases, the preclinical research of drugs against these diseases, and their experimental prevention can thus be elicited *in vivo*. Unfortunately, currently, potential applications of the tree shrews as models mentioned above are limited because the tree shrews cannot be bred on a large scale in cages.

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