

D-galactose-induced animal model of male reproductive aging

Evy Sulistiyoningrum*¹

¹Histology Department Faculty of Medicine Islamic University of Indonesia

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ABSTRACT

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*Corresponding author:

evysulistiyoningrum@gmail.com

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Aging is a complex biological process involving molecular, cellular and organic changes. Aging process is not merely caused by chronological age but it can be accelerated by environmental factors contributes to oxidative stress. Aging in male reproductive system is characterized by many conditions such as testicular atrophy, decreases testicular function of spermatogenesis, decreased testicular function of testosterone production which can lead to a serious clinical condition, infertility. Management of male infertility with excellent outcome is still a challenge and therefore new strategies need to be examined. Because of difficulties and ethical issues in human research, animal models of male reproductive aging are useful research tools for this purpose. Rodent models of male reproductive aging are the first choice because similarity of metabolism system and short lifespan. D-galactose animal model for aging seems to be closer to human aging of male reproductive system. It is an advantageous model for testing potencies of pharmacological agent and natural compounds on aging.

Penuaan adalah proses biologis kompleks yang melibatkan perubahan di tingkat molekuler, seluler dan organisme. Proses penuaan tidak hanya disebabkan karena usia kronologis tetapi juga dapat dipercepat oleh faktor lingkungan yang berhubungan dengan stres oksidatif. Penuaan pada sistem reproduksi pria ditandai dengan berbagai perubahan yang dapat berupa atrofi testikular, penurunan fungsi testikular dalam melakukan spermatogenesis, penurunan fungsi testikular dalam memproduksi hormon testosteron yang dapat berakibat pada suatu kondisi klinis serius yaitu infertilitas. Penatalaksanaan paripurna kasus infertilitas pada pria sampai sekarang masih menjadi tantangan sehingga masih diperlukan penelitian untuk menemukan strategi-strategi baru penatalaksananya. Karena keterbatasan penelitian serta masalah etik pada penelitian dengan subyek manusia, model hewan coba pada penuaan di sistem reproduksi pria menjadi perangkat penelitian yang sangat bermanfaat. Model hewan coba dengan rodensia menjadi salah satu pilihan utama karena memiliki sistem metabolisme yang mirip dengan manusia dan lama hidup yang pendek. Hewan model penuaan terinduksi D-galaktosa memiliki kesamaan dengan penuaan pada sistem reproduksi pria. Metode ini dapat menjadi salah satu metode yang bermanfaat untuk melakukan pengujian potensi agen farmakologis dan bahan alam terhadap penuaan.

INTRODUCTION

Increasing modernization, increasing life expectancy and many social factors worldwide are accompanied by an increasing trend of delayed parenthood in developed countries and developing countries as well.¹ Increasing

chronological age (i.e. aging) can affect male reproductive system. Accumulation of reactive oxygen species (ROS) and the loss of telomerase activity that accompanies aging can reduce male reproductive system performance.² On the other hands, many environmental and also nutritional

factors were known to provoke generation of ROS and oxidative stress resulting on aging acceleration.³ Aging in male reproductive system can result in infertility.¹ Infertility is the inability to contribute conception after 12 months of unprotected intercourse; this may be due to male, female or some unexplained factors. Infertility is a major clinical problem, affecting people medically, psychologically and socially. About 15% of all couples in the US are infertile.⁴ Male infertility alone contribute approximately 35% to 50% of infertility cases.⁵

Medication currently available for improving sperm quality and recommended for treating men with infertility is oral antioxidants. Several researches reported that oral antioxidant and oral-antioxidant-related products (e.g., vitamin E, vitamin C, selenium, quercetin, folate, zinc and carnitine) improve sperm quality (sperm count, motility, viability and morphology) and pregnancy rates. However, studies investigating potential therapeutic effect of such products have reported conflicting results.⁶

Because of difficulties and ethical issues in human research, animal models of male aging are useful and essential for understanding the pathogenesis of alterations, and the utility of therapeutic agents in fertility parameters. In an animal model, alteration or phenomenon in one or more aspects resembles the same condition human. Rodent models of aging are the first choice and are most widely used, as they share many similarities with human. The aim of this study is an overview one of the most frequently-used model for generating animal model of male reproductive aging, induced by D-galactose considering its advantages and mechanism of action for male reproductive aging research in humans.

AGING AND MALE INFERTILITY

Pathogenesis of male infertility is multifactorial, and any alteration to normal physiology of reproductive organs may affect sperm functions that causes problem for a successful fertilization.⁷

Besides, aging males also acquire hypogonadism characteristics because of relatively low testosterone serum levels and certain changes in the testis, which are coupled with the loss of gonadal endocrine function and fertility.⁸ Cocuzza et al. reported that in aged person there were decreased semen quality (reduced total sperm count, concentration and motility), and decreased normal morphological sperm ratio and DNA-fragmented sperm.² Sunanda et al. reported that age was negatively correlated with percentage of sperm with progressive motility, vitality and normal morphology.⁹

Furthermore, the effect of aging on reproductive organs can be also observed in testicular and epididymal morphology. In advanced ages, there was a degenerative changes in testicular volume. There were negative associations between increasing paternal age and reduction in testicular volume up to 31% and reduction testicular size.¹⁰ This decreased testicular volume is also attributed to the decrease in number of spermatogenic and Sertoli cells. Histologically, degenerative changes in germinal epithelium and decreased number of Leydig cells can be observed.¹¹ Xu et al reported testes of aged individual showed vacuolation of seminiferous (85% of incidence) (Figure 1, upper right picture) and abnormal changes in interstitial compartment contributed in decreased testicular function.¹² Another obvious phenomenon was the heterogeneity of spermatogenesis, which reflected the progressive degenerative process of testicular tissues. Interstitial compartment showed loose connection and Leydig cells aggregations and reduced number, which resulted in reduced secretion of testosterone (Figure 1). On the other hand, in the epididymis, an important structure for sperm full maturation, aging induces apoptosis and increases HSP70 stress protein in the epididymis.¹³

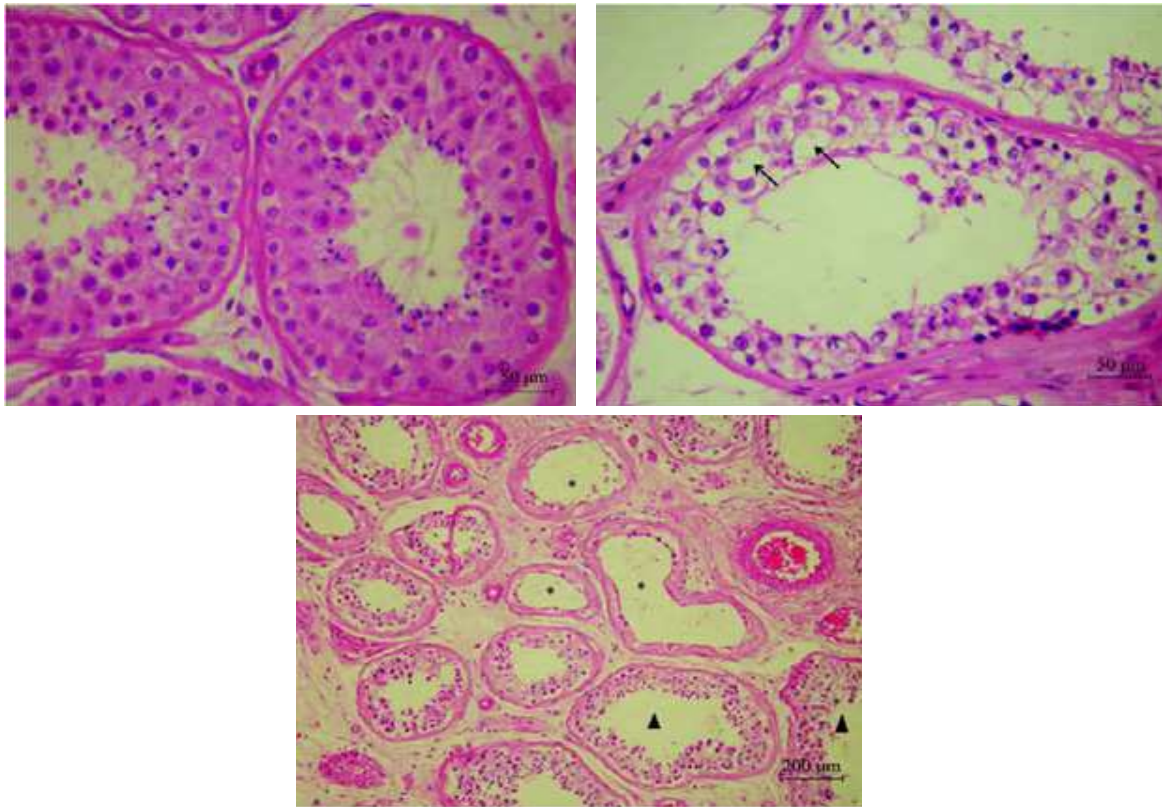


Figure 1. Comparison of seminiferous tubule.¹² Normal seminiferous tubule in young adult showing optimal spermatogenesis. All stages of spermatogenesis evident (upper left). Abnormal seminiferous tubule taken from aged person showing abnormal spermatogenesis (stop at spermatogonium stage) and vacuolization (arrows) (upper right). Low magnification of testicular biopsy showed many tubules with inadequate spermatogenesis (asterisks) but some with normal spermatogenesis (arrowhead) and accumulation of loose connection tissue in interstitial (lower picture); Hematoxylin Eosin stain.¹²

Aging also affect endocrine function of testis. It has been shown that senescence is associated with the fall in serum levels of androgen and increase in tissue oxidative damage.¹³ Decreased number of Leydig cell might be the main cause of this condition. Free and bound testosterone levels decline with senescence in men. The causative mechanism of this phenomenon may be derived from the hypothalamic-pituitary-testicular axis. Further, it has been shown that luteinizing hormone (LH) levels have a reverse relationship with serum testosterone levels in the case of aging.¹⁰

The most scientists approve the free radicals/oxidative stress theory that underlying many conditions related to aging process. This theory states that the age-related accumulation of free radicals and superoxide leads to damage of macromolecular components such as

carbohydrates, lipids, proteins and nucleic acids which are the building blocks of cellular structure.³ The most common reactive oxygen species (ROS) that have potential effects on the reproductive system are superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). Further, mammalian spermatozoa have a large amount of phospholipids, sterols, and saturated and polyunsaturated fatty acids, which makes them susceptible to ROS-mediated damage.¹⁴

Aging molecular mechanism also can be explained by various molecular mechanisms such as DNA mutations and chromosomal aberrations and epigenetic patterns. This molecular aging process was shown to induce changes in reproductive hormone's profiles, decrease sperm parameters and contribute to male infertility.¹⁰

ANIMAL MODEL OF MALE AGING REPRODUCTIVE SYSTEM

The ideal condition for mimicking natural aging in human is studying natural aged animal. But, problems concerning with laboratory aging animal models is determining animal life span. In term of laboratory animal, we deal with some domesticated animals which can attain longer or shorter lives than if they were living in their wild habitat. The life span of a laboratory animal is determined by factors such as species-specific genes and environmental factors. Humans are not inbred and live in remarkably different conditions from both environmental and socio-anthropological-cultural points of view.¹⁵

Therefore, many models to induce or accelerate aging process were performed in animal for mimicking aging process in male reproductive system. Some methods are based on generation of oxidative stress condition *in vivo*. In this oxidative stress, accumulation of ROS can induced by several methods including partial orchidectomy, inducing diabetes or administration of arsenic, lead, D-galactose etc. These methods generate suppression on antioxidant defense system leading to oxidative damage to cellular macromolecules including DNA, protein and lipids. This methods also produce tissue damage, altering biochemical compounds and surrounding cell membranes.¹⁶ This methods interfere with metabolism of essential antioxidant molecules responsible for metabolism and excretion of xenobiotics.¹⁷ Alteration in male organ system (e.g male reproductive system) can be performed via suppressive influence on cellular componen involving in spermatogenesis and an androgenesis in male reproductive system. Furthermore, they can causes testicular toxicity probably by affecting the pituitary testicular axis.¹⁸

Another method for mimicking conditions of human reproductive system aging is by giving anti-androgen treatment. Agents work in such mechanism is flutamide (4'-nitro-3'-trifluoro-methylisobutyranilide). Flutamide acts by inhibiting uptake and/or binding of

dihydrotestosterone to the target cell receptor, thus interfering with androgen action. In male patients with androgen excess, flutamide is used therapeutically to treat androgen dependent prostate cancer.¹⁹ Flutamide is regarded as a model anti-androgen. Several studies demonstrated the effects of short-term androgen blockage induced by the administration of flutamide to immature or mature males.²⁰

MALE REPRODUCTIVE SYSTEM IN D-GALACTOSE INDUCE ANIMAL MODEL

The most prevalent animal models used for investigating male reproductive system aging involve using D-galactose -induced rat and mouse or models. D-galactose induction can produce animal aging condition in stabile, simple and economics.²¹ Recent reports implicate the chronic administration of D-galactose in accelerating aging in mice.²² A chronic administration with a low dose of D-galactose induces changes that resemble natural aging, such as a shortened lifespan, cognitive dysfunction and neurodegeneration, oxidative stress and advanced glycation endproduct (AGE) formation and gene transcriptional changes.²³⁻²⁵ D-galactose-induced senescence acceleration has been widely used as a aging model for studying aging mechanisms and screening drugs.²⁴

However, studies on male reproductive aging in D-galactose-induced mouse models have been reported only in recent years. Liao et al. reported 8 weeks exposure of D-galactose increased testicular lipid peroxidation, decreased ratio of testis weight/body weight and sperm count, percentages of both immotile sperm and abnormal sperm increased compared to the control group.²² Furthermore, D-galactose-treated groups exhibited RNA transcripts of nine spermatogenesis-related genes (Cycl2, Hk1, Pltp, Utp3, Cabyr, Zpbp2, Speer2, Csnka2ip and Katnb1) that were up-regulated or down-regulated by at least two-fold compared to the control group. Several of these genes are critical for forming sperm-head morphologies or maintaining nuclear integration (e.g., cyclin, basic protein of sperm head cytoskeleton 2

(Cylc2), casein kinase 2, alpha prime interacting protein (Csnka2ip) and katanin p80 (WD40-containing) subunit B1 (Katnb1).

Ahangarpour et al. used a D-galactose-oral gavaged mouse model for 6 weeks and reported decreased in testicular volume and size, decreased sperm counts decreased testosterone level and increased serum LH (Luteinizing Hormone) levels.²⁶ Consistent with Ahangarour, Salman et al. reported treatment with D-galactose for 6 weeks

had toxic effect fo testis evidenced by decreased epididymal sperm parameter (sperm count, percentage of normal morphology, percentage of normal motility and viability). Futhermore, a decreased in serum testicular level and increased serum level of LH, decreased on superoxide dismutase (SOD) activity and increased activity of lactate dehydrogenase indicating that chronic administration of D-galactose had toxic effect on sperm via oxidative stress mechanism.²⁷

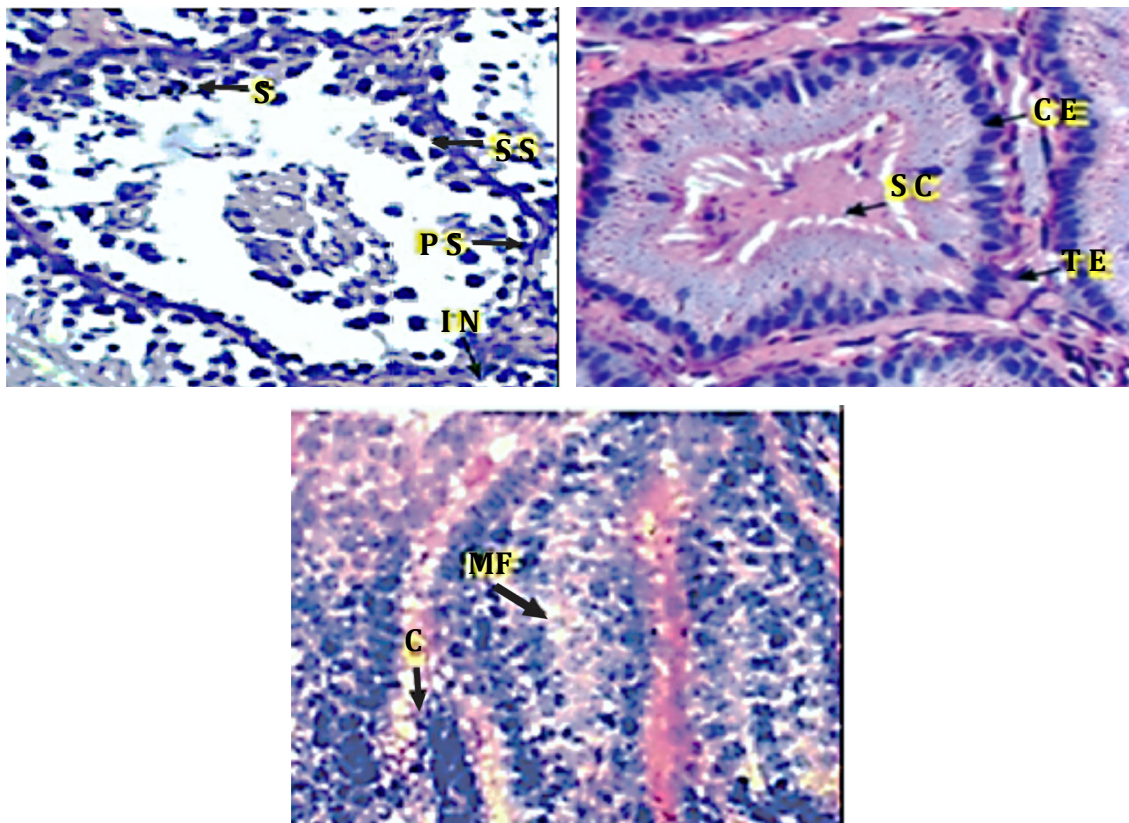


Figure 2. Histological changes of male reproductive organ after D-galactose-treatment.²⁸ A (Upper left): Seminiferous tubule showed disturbed epithelial structure with decreased number of spermatogenic cell. B (Upper left). Epididymis showed lost structural integrity, and abnormal epithelium. C. (Lower picture) Seminal vesicle showed lost the structural integrity, disappearance of the muscle layer, abnormal glands' cells. Higher magnification, Hematoxylin Eosin stain (PS: Primary spermatocytes, SS: Secondary spermatocytes, S=sperm= IN = Intertitial, TE: Tubules of epididymis, CE: Columnar epithelium, SC: Stereocilia, MF: Mucosal fold, C: Crypts).

Shaikh et al. reported a decreased in masses of testes, epididymis, and seminal vesicle was observed in D-galactose treated mice.²⁸ The decreased might be due to reduced tubular size, spermatogenic arrest and inhibition of steroid biosynthesis of Leydig cell. Decreased testicular weight is related to spermatogenic failure as

suggested by concomittant decreased epididymal sperm count. The weight loss of accessory sex organs may be due to reduced bioavailability of sex hormones. Histologically, D-galactose treated seminiferous tubules had disturbed epithelial structure and their number was also decreased, spermatogenic cells number, especially number

of spermatocytes was decreased (Figure 2). In the interstitial compartment, enlarged congested blood vessels and cellular exudates in the interstitial cells were observed. Epididymis of D-galactose treated group showed lost of structural integrity, decreased number of sperm in the lumen and disappeared of muscle layer in adventitia. Furthermore, the seminal vesicle structure also abnormal, the vesicle lost the structural integrity, disappearance of the muscle layer and cuboidal cells (Figure 2).

ACTION MECHANISM OF D-GALACTOSE

The exact mechanism of cellular senescence induced by galactose remain unclear. D-Galactose is a physiological nutrient and a reducing sugar that reacts with free amines of amino acids in proteins. D-Galactose is normally metabolized by galactose-1-phosphate uridylyltransferase and D-galactokinase. One possible mechanism is that excess intracellular galactose makes cells to

rely more heavily on mitochondrial respiration for energy production. This condition can lead to overproduction of ROS and induction of senescence.²⁹ Another theory postulates that galactose can bind with protein to form AGEs through nonenzymatic glycation. As such, oversupply of D-galactose could contribute to generation of ROS through oxidative metabolism of D-galactose as well as through glycation end products.²⁵ When the concentration of D-galactose exceeds normal levels, it is converted to aldehydes and H_2O_2 . A large dose of D-galactose also can be converted to galactitol and cause an osmotic stress, generating reactive oxygen species (ROS).³⁰ In cellular level, D-galactose was reported to enhanced Spinster-induced senescence of the human fibroblast cell and this synergism required the transporter activity of Spinster (a putative lysosomal carbohydrate efflux transporter).³¹

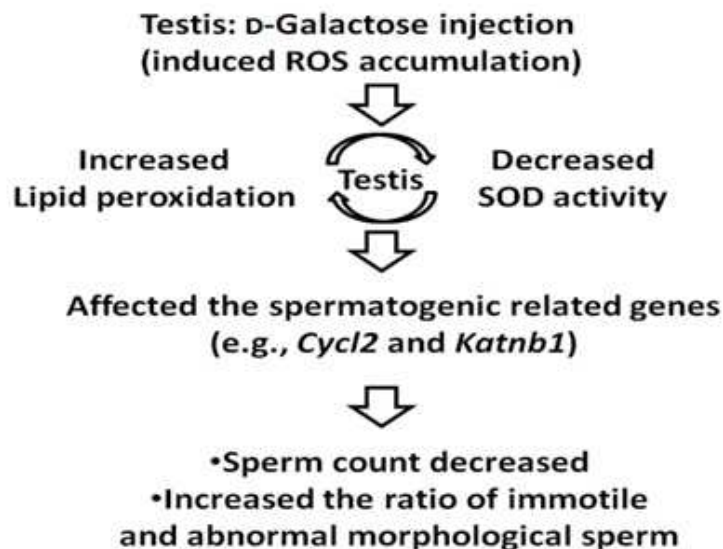


Figure 3. Mouse model of male reproductive aging induced by D-Galactose injection²²

Considering effect on male reproductive system, Liao et al. postulated the mechanism of D-galactose in mouse model of male reproductive system (Figure 3).²² Previous studies reported that D-galactose forms AGEs in vivo and accelerate the aging process. The D-Gal-injected mice demonstrated decreased SOD activity and

increased lipid peroxidation amount.²⁷ Low SOD activity reflects normal male aging.³² Further, SOD accumulation affects the expression of spermatogenic genes (e.g., *Cycl2* and *Katnb1*) and results in the decreased sperm count and increased ratio of immotile and abnormal sperm morphology.²²

METHODS FOR INDUCING AGING WITH D-GALACTOSE

Previous studies reported effectivities of D-galactose in mimicking aging in human. However, studies on male reproductive aging in D-galactose-induced mouse models have been reported only in recent years. Some studies focused on optimizing D galactose in male reproductive system while other studies tested potency of substances in reducing the effect on male reproductive system.

D-galactose ($C_6H_{12}O_6$) had 180,16 g/mol molecular weight and can be administered orally or parenterally in various dose and duration of administration (Table 1). These administration can induced aging in multiple organ system. Oral administration of D-galactose was easy to given and induced less harm for the animal. Dose given to induce alteration in biochemical male reproductive system indicators such as decreased sperm parameters via oral gavage varied between 3 mg to 40 mg/kg, while Ahangarpour used 500 mg/kg to induce alteration in testicular weight and volume.²⁷ D-galactose could be solved in distilled water and given with oral gavage of 1 to 5 ml volume for 6 weeks. Parenteral administration can be given via intraperitoneal injection, and subcutaneous injection. The method was high reproducible, however, this is quite difficult and needs experience. Duration of administration varied between 15 days to 56 days. In parenteral administration, D-galactose could be solved in sterile water for injection or phosphate-buffered saline. Dose given to induce alteration in biochemical male reproductive system indicators such as decreased sperm parameters via parenteral administration is 100-200 mg/kg, up to 1 ml solution. Abnormal changes in male reproductive system could be in biochemical parameters (gonadal or pituitary hormones), oxidative stress markers (SOD), Malondealdehyde (MDA), Lactate Dehydrogenase or Lipid Peroxidation derived from serum or tissue homogenate), sperm parameter (sperm count, percentage of normal sperm morphology, motility and viability), morphometric changes (volume, size, weight) or histological changes.

CONCLUSION

In conclusion, although none of the animal models of aging represents complexity aging of the human, it seems that D-galactose model which seems to be closer to human aging of male reproductive system is an advantageous model for testing potencies of pharmacological agent and natural compounds on aging. There are great variation of dose, duration of administration and route of administration that may explain some differences observed in different studies.

Table 1. Different protocols used in the selected studies for induction male reproductive system aging with D-galactose

Subjects Character	D-galactose Dose	Route of administration	Duration	Alteration	Ref.
C57Bl/6 mice	100 mg/kg	intraperitoneal injection	6 weeks	Increased SOD serum level, decreased testicular weight and sperm parameter	(22)
	100 mg/kg	intraperitoneal injection	8 weeks	Decreased testicular weight, decreased sperm parameter	
	200 mg/kg	intraperitoneal injection	6 weeks	Decreased sperm parameter	
	200 mg/kg	intraperitoneal injection	8 weeks	Increased MDA serum level, decreased testicular weight and sperm parameter	
Albino rats, 200–250 grams	3 mg/kg	oral gavage	6 weeks	Decreased sperm parameter and testosterone level, increased LDH and MDA, and LH level	(27)
	10 mg/kg	oral gavage	6 weeks	Decreased sperm parameter, increased MDA and LH level	
	20 mg/kg	oral gavage	6 weeks	Decreased sperm parameter, increased LDH, MDA and LH level	
	30 mg/kg	oral gavage	6 weeks	Decreased sperm parameter, increased LDH level	
Swiss albino mice, 6 months, 50-55 grams	40 mg/kg	oral gavage	6 weeks	Decreased sperm parameter and SOD level	(28)
	5% D-galactose 0,5 ml	subcutaneous injection	20 days	Decreased sperm count and relative organ weight, histological changes of testis, epididymis and seminal vesicles	
Naval Medical Research Institute mice, 3 months, 20-25 grams	500 mg/kg	oral gavage	6 weeks	Decreased testis weight and volume, increased gonadotropin level, decreased testosterone level and sperm count	(26)
Albino mice, 5-6 months, 40- 50 grams	5 % D-galactose 0,5 ml	subcutaneous injection	15 days	Increased testicular and epididymal MDA level, increased mitochondrial peroxidation, decreased sperm count, degenerative seminiferous tubule epithelium	(33)

MDA = malonaldehyde, SOD = superoxide dismutase, LH = luteinizing hormone, LDH = lactate dehydrogenase

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