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Mesenchymal Stem Cell Derived Microvesicles Versus Ozone in Ameliorating Testicular Changes in Hypothyroidism Adult Albino rat

Magdy F. Gawish, Samia A. Abd EL-Baset, Salma S. Shalabi & Nahla E. Ibrahem

Medical Histology and Cell Biology Department, Faculty of Medicine, Zagazig University, Egypt

Abstract

Hypothyroidism is a clinical disorder commonly encountered by the primary care physician. Untreated hypothyroidism can contribute to hypertension, dyslipidaemia, infertility, cognitive impairment, and neuromuscular dysfunction. In the past decades, clinical studies revealed that the thyroid hormones play a mandatory role in spermatogenesis and steroidogenesis. The efficiency of spermatogenesis is directly correlated to the number of functional Sertoli cells entrenched during adulthood (Sharpe 2010). In addition; it is now known that T3 regulates the testicular growth and maturation in different mammals. Deficiency of thyroid hormones production causes other serious health defects that may lead defect in kidney growth and function, multiple heart diseases, and myocardial infarction. Medical ozone is a pharmaceutical compound which consists of a mixture of gases containing not less than 5% of ozone (O3) and not more than 95% of pure oxygen (O2). It has the ability to protect the body against pathological conditions caused by oxidative stress. The role of MVs (from oviduct or seminal plasma) in sperm maturation, motility as well as its membrane integrity and function has been discussed.

Keywords: Ozone, Mesenchymal Stem Cell Derived Microvesicles, Testicular Changes, Hypothyroidism.

Full length article **Corresponding Author*, e-mail*: shalabisalma92@gmail.com*

1. Introduction

The testes are paired ovoid gland weighing about 15gm in human adult male, located in the scrotum outside the abdominal cavity. The adult testes produce sperm, also contain endocrine cells secreting hormones such as testosterone, which drives male reproductive physiology [1-2]. Embryologically, in human, both male and female embryos have two pairs of genital ducts: mesonephric (wolffian) ducts and paramesonephric (müllerian) ducts. The default sexual differentiation is female. However, having the sex-determining region (SRY) gene, defines the male reproductive system and development of the testes [3]. Kashimada and Koopman [4] mentioned that SRY is a transcription factor that acts in conjunction with the autosomal SRY-Box Transcription Factor 9 gene (SOX9), a transcriptional regulator, which can also induce testes differentiation. SOX9 is known to bind to the promoter region of the gene for antimüllerian hormone (AMH) and probably regulates this gene's expression. The testes incubate Sertoli cells, which produce Mullerian inhibitory substance (MIS) to induce regression of the Mullerian ducts, which form the female reproductive tract. Also the Sertoli cells regulate the development of seminiferous tubules. The testes also, develop Leydig cells that produce testosterone, the major driver of male reproductive development [3].

The testes develop near the kidneys in the posterior portion of the abdomen, and they usually begin their descent with the first part of its duct system, blood vessels, lymphatics and nerves into the scrotum through the inguinal canals (pathways in the anterior abdominal wall) during the latter half of the seventh month of fetal development [5]. Anatomically in human, each testis in scrotum measuring about 5cm long, 2.5cm in diameter and has a mass of 11–17 gm with the right one usually slightly larger and heavier than the left one. While in laboratory rats testis is about 20 mm in length and 14 mm in diameter and the average weight is about 6 gm each, or 4.4% of their body weight [6]. Each testis suspended within the end of an elongated musculofascial pouch, which is continuous with layers of the anterior abdominal wall and projected into the scrotum. Testes are connected by the spermatic cords to the abdominal wall and tethered to the scrotum by scrotal ligaments which is a remnants of gubernaculum Fig. B [1]. During migration, the testis carries with it an investing layer of peritoneum. So that, in the scrotum the testis is almost completely surrounded by a double layer of mesothelium, enclosing a potential space.

This double lining is called the tunica vaginalis and, consists of visceral and parietal layers, separated by a thin layer of serous fluid. The fluid is secreted by the mesothelial cells and acts as a lubricant, allowing testis to move freely in the scrotal sac [7]. Histologically, the testes are surrounded by a capsule called the tunica albuginea; which is a white fibrous capsule composed of dense irregular connective tissue partially covers the testes internal to tunica vaginalis. The tunica albuginea, gives rise to numerous incomplete collagenous septa which divide each testis into about 250 testicular lobules. Within each lobule, there are one to four highly convoluted tubes, called the seminiferous tubules, in

which spermatozoa are produced, each tubule is about 150 micrometer (µm) in diameter and 80 µm long; it is U-shaped with the two ends opening in the rete testis [5]. The seminiferous tubule consists of a central lumen lined by a specialized seminiferous epithelium containing two distinct cell populations: The somatic Sertoli cells and the spermatogenic cells (spermatogonia, spermatocytes, and spermatids) [8]. The seminiferous epithelium is encircled by a basement membrane and a wall formed by collagenous fibers, fibroblasts, and contractile myoid cells.

Myoid cells are responsible for the rhythmic contractile activity that propels the nonmotile sperms to the rete testis. Sperms acquire forward motility after they have passed through the epididymal duct [9]. The seminiferous epithelium can be classified as a stratified epithelium with rather unusual characteristics not found in any stratified epithelium of the body. In this stratified epithelium, somatic columnar Sertoli cells interact with mitotically dividing spermatogonia, meiotically dividing spermatocytes, and a haploid population of spermatids undergoing a differentiation process called spermiogenesis [10]. By mitotic division, spermatogonia give rise to primary spermatocytes, which are the largest germ cells of the germinal epithelium. The primary spermatocytes have relatively large nuclei and are in the middle third of the seminiferous epithelium. After 10-22 days, these cells undergo first meiotic division and give rise to smaller secondary spermatocytes, which rapidly undergo a second meiotic division with no Deoxyribonucleic acid (DNA) replication [9]. Spermatids, known as early and late, the early spermatids with a round light nucleus, have a diameter of about 9 µm and a haploid chromosome number and DNA content.

While, the late spermatids have cylindrical-shaped condensed nuclei and embedded in invaginations of Sertoli cells. While spermatids move toward the tubule lumen, they elongate and undergo an elaborate process of maturation into spermatozoa without mitosis, known as spermiogenesis. Then, spermatozoa are released into the lumen and carried into efferent ducts [11]. Sequential changes during spermiogenesis take place in the upper layers of seminiferous epithelium whereby spherical, nonmotile spermatids become elongated and motile spermatozoa. These changes include condensation of nuclear chromatin, elongation of the nucleus, formation of the acrosome, migration of cytoplasmic organelles to positions typical of mature cells, formation of a single flagellum and loss of residual cytoplasm. Spermatozoa, about 300 million being produced daily, are released into the lumen [12]. At first, several small acrosomal vesicles consist of the juxtanuclear Golgi complex coalesce into a single large membrane-bound acrosome, which adheres to the nuclear envelope. An electron-dense acrosomal granule forms within the vesicle, which gradually spreads to cap the anterior surface of the nucleus and ultimately becomes the front of the mature spermatozoon.

The acrosome, a modified lysosome, contains hyaluronidase, lysosomal hydrolases, and protease enzymes that allow spermatozoa to penetrate the corona radiate and zona pellucida of oocyte during fertilization [13]. At a later stage of spermiogenesis, a pair of centrioles migrates to the opposite pole of the spermatid nucleus, and a single flagellum grows out from one. Its core has an axoneme with two central microtubules and nine peripheral microtubular doublets, which provides substrate for sperm tail motility. Mitochondria migrate toward the flagellum and form a sheath or collar around it. Residual cytoplasm and redundant organelles are shed and phagocytosed by adjacent Sertoli cells [14]. The highly specialized spermatozoa are about 60 µm long and are typically divided into five distinct regions. A small, condensed conical nucleus in the head piece with the acrosome; a centriole pair occupies the neck piece. A middle piece contains helically arranged mitochondria that provide energy to propel the spermatozoon. The last two regions principal and end pieces contain the axoneme surrounded by coarse longitudinal fibers Fig. D [15].

Sertoli (sustentacular) cells play a critical role in support and maturation of spermatozoa. These columnar cells, with indistinct borders, extend from the basement membrane to the lumen of the seminiferous tubule. Their apices have crypt-like recesses that hold spermatids until release of newly formed spermatozoa into the lumen [11]. After puberty, Sertoli cells constitute about 10% of cells in the seminiferous epithelium. They have pointed euchromatic nuclei with prominent nucleoli. Their cytoplasm contains microtubules and intermediate filaments forming a prominent cytoskeleton, as well as long, slender mitochondria, a conspicuous smooth endoplasmic reticulum, large numbers of lipid droplets and lipofuscin-laden lysosomes [14]. In addition, Sertoli cells phagocytose spermatid remnants and secrete fluid and many substances, as androgen-binding protein, which is essential for spermatozoa survival. The extensive cytoskeletal network of Sertoli cells helps provide for spermatozoa movement as cell junctions are closely related to actin filaments and endoplasmic reticulum at sites called ectoplasmic specializations, which may adjust to changes in junctional architecture as spermatozoa move toward the lumen [14].

Leydig cells are clusters of large polyhedral, approximately 15 µm in diameter eosinophilia cells in loose connective tissue between seminiferous tubules. They have foamy, washed-out cytoplasm due to high lipid content, as they store cholesterol for synthesis of testosterone. These cells have an eccentric spherical nucleus, although occasionally they may be bi nucleated with one or two prominent nucleoli, and cell surfaces have numerous small microvilli [1]. Also, Leydig cells lie close to fenestrated capillaries and small lymphatic vessels. They have features typical of steroid secreting cells as their cytoplasm contains abundant, tightly packed smooth endoplasmic reticulum (SER), relatively few ribosomes and rough endoplasmic reticulum, numerous scattered mitochondria with tubulovesicular cristae, a large juxtanuclear Golgi complex and many spherical lipid droplets of various sizes also occupy the cytoplasm. Rod-like crystalloid cytoplasmic inclusions (crystals of Reinke) possessing highly ordered pattern of internal structure in Leydig cells, but their function remains unknown. These inclusions are not present before puberty and are most common with advancing age.

The amount of lipofuscin pigment associated with tertiary lysosomes also increases in old age [1]. In the human, the interstitial compartment represents about 12–15% of the testicular volume. While in laboratory rats, presenting rather small testes, the interstitial compartment is comparably sparse and comprises small groups of Leydig cells gathering around blood vessels [16]. Physiologically, normal function of the male reproductive system depends on both hormonal and neural mechanisms. Hormones are primarily responsible for the development of reproductive structures and maintenance of their functional capacities, the development

of secondary sexual characteristics, the control of sperm cell formation, and influence over sexual behavior. Neural mechanisms are primarily involved in sexual behavior and control of the sexual act. Regulations of sex hormone secretion mechanisms that influence the male reproductive system involve the hypothalamus, the pituitary gland and the testes. A small peptide hormone called gonadotropinreleasing hormone (GnRH), or luteinizing hormone releasing hormone (LHRH), are released from neurons in the hypothalamus.

GnRH passes through the hypothalamohypophyseal portal system to the anterior pituitary gland. In response to GnRH, cells within the anterior pituitary gland secrete two hormones, referred to as gonadotropins because they influence the function of the gonads [13]. Luteinizing hormone (LH) in males, which sometimes called interstitial cell–stimulating hormone (ICSH) binds to the interstitial cells in the testes and increases their rate of testosterone synthesis and secretion. While, follicle stimulating hormone (FSH) binds primarily to Sertoli cells in the seminiferous tubules and promotes sperm cell development. Testosterone is the major male hormone secreted by the testes. It is classified as an androgen (andros is Greek for male human being) because it stimulates the development of male reproductive structures. Nearly all of the androgens, including testosterone, are produced by the interstitial cells, with small amounts produced by the adrenal cortex and possibly by the Sertoli cells [3]. Testosterone enters cells of target tissues where it may remain intact or be converted to dihydrotestosterone (DHT) by a 5-alpha reductase enzyme.

DHT responsible for male hair pattern (facial, axial and pubic hair), including the pathology of male pattern balding, and increased sebaceous gland secretion and acne. Also, the testes secrete small amounts of estrogen and progesterone. Many clinical studies revealed that the thyroid hormone plays a mandatory role in spermatogenesis and steroidogenesis. Those studies revealed that triiodothyronine (T3) regulates the testicular growth and maturation, in different mammals. This can occur through the well-known physiological role of thyroid hormones in modulating process of oxidative stress caused by reactive oxygen species [17]. The butterfly-shaped thyroid gland is located just inferior to the larynx. It is composed of right and left lateral lobes, one on either side of the trachea, that are connected by an isthmus anterior to the trachea. About 50% of thyroid glands have a small third lobe, called the pyramidal lobe that extends superiorly from the isthmus [5]. Microscopically, Menzilcioglu et al. [18] described that the thyroid gland has a thin capsule of connective tissue, which extends into the glandular parenchyma and divides each lobe into irregularly shaped and sized lobules.

The functional units of the thyroid are follicles, which are spherical sacs between 20 and 900 µm in diameter. The wall of each follicle consists primarily of cells called follicular cells. Follicular cells have a round to ovoid nucleus with two nucleoli and basophilic cytoplasm. These cells also have numerous apically located lysosomes, rough endoplasmic reticulum (RER), rod-shaped mitochondria, and a supranuclear Golgi complex. Numerous small vesicles, dispersed throughout the cytoplasm, are believed to contain thyroglobulin that was packaged in the Golgi complex and is destined for exocytosis into the follicle lumen. Thyroid parenchyma also contains C (clear) cells, so-called because they have pale-staining cytoplasm, which is more pronounced in some species than in the human thyroid. C cells are members of the amine precursor uptake and decarboxylation (APUD) system of dispersed neuroendocrine cells. Occasionally they occur in clusters in the interfollicular stroma, which is why they are also called parafollicular cells. They produce hormone calcitonin, which regulates calcium homeostasis [19]. Thyroid gland releases T3 and thyroxine (T4). These hormones may modulate male reproductive functions by various routes, which have been research interests since past several decades. In addition; T3 regulates testicular growth and maturation in different mammals [20].

2. Testicular Changes in Hypothyroidism

Hypothyroidism is one of the most common thyroid disorders in humans. Hypothyroidism is a pathological condition of thyroid hormone deficiency. It can be identified as obvious hypothyroidism when T3 and T4 serum levels are reduced, and the Thyroid-stimulating hormone (TSH) level is elevated which can lead to serious adverse health effects. Hypothyroidism is provoked by carbimazole; the most frequently and preferentially used drug in treatment of hyperthyroidism. The drug is given to the animals through a stomach tube so the dose should accurately adjusted. Carbimazole is a 3-carbethoxy methimazole derivative, metabolized to methimazole in the liver. Methimazole reduces the production of serum thyroxine, thyroid-stimulating hormone and thyrotropin-binding inhibitory immunoglobulins [21]. Many studies reported that drugs inducing hypothyroidism can cause oxidative stress that lead to severe cellular damage in many tissues including; thyroid, liver, kidney, lung, pancreas, and gastric mucosa and salivary gland tissues. Moreover, hypothyroidism might lead to an impairment of reproductive efficiency of adult male rats [22].

2.1. Role of ozone and and Mesenchymal Stem Cell Derived Microvesicles in Ameliorating Testicular Changes in Hypothyroidism

Various studies showed that medical ozone treatment can improve the testicular destruction by chemotherapeutic drugs. And like antioxidants, it can regulate the antioxidant defense system of cells by elevating enzymatic and non-enzymatic antioxidants capacity of the testicular tissue and protected them from damages induced by ischemia reperfusion injury after torsion/detorsion of testes [23]. Ozone (O3) is a gas made of three atoms of oxygen (O) with a cyclic structure. It is a highly water-soluble inorganic and an unstable molecule due to its mesomeric state, means that it has a characteristic of different functional groups in a chemical compound. The naturally occurring ozone is present in the stratosphere surrounding the earth and protects humans from the dangerous ultraviolet radiations. Ozone can be produced by medical generators from pure oxygen after passing through a high voltage gradient. Medical ozone is a mixture of gases containing not less than 5% of ozone and not more than 95% of pure oxygen (O2). Because of its instability and short half-life (40 min at 20°C), ozone cannot be stored in tanks [24]. Despites, difficulties with its storage, ozone therapy has been given in medical practice via several routes that include transdermal, intramuscular, rectal, nasal, oral, vaginal, intravenous, intra-arterial, intraperitoneal, intra-pleural, topical, dental, intra-discal and by auto-hemotherapy.

Auto-hemotherapy or self-blood therapy, a method in which a patient's blood is withdrawn, and then, the blood is mixed with a combination of saline and medical-grade ozone gas then re-injected directly back into the vein of the

patient through an intravenous drip over several minutes. The aim of this therapy is to enhance patient's immune system to fight disease [25]. Ozone has used to treat several pathologies such as chronic cutaneous ulcers, peritonitis, infected wounds, ischemic diseases, and joint problems. Furthermore, ozone has strong bactericidal, antiviral, anti-fungal and anti-protozoal actions as well as other therapeutic effects. In recent years, ozone therapy has shown to exhibit positive effects on wound healing and pathological conditions, such as age-related macular degeneration, ischemic and infectious diseases [26]. Extracellular vesicles (EVs) are a family of particles released from cell that are an important intercellular communication system facilitating the transfer of macromolecules between cells. They contain abundant contents such as proteins and nucleic acids can be transferred b/w cells to regulate homeostasis of recipient cells [27]. EVs include exosomes, microvesicles also known as shedding vesicles, ectosomes, nanoparticles and apoptotic bodies. They are generally distinguished by their size, surface markers and biogenesis.

These particles have been found in most of body fluids. Exosomes are vesicles with a diameter of 20–100 nm, which originate from budding of late endosomes, whose lumen becomes full of IntraLuminal Vesicles (ILVs). For this reason, these endosomes are called MultiVesicular Bodies (MVBs). Upon MVB fusion with plasma membrane through exocytosis, ILVs released extracellularly, taking name of exosomes [28]. Exosomes are shed into extracellular space constitutively or as consequence to physical or chemical stress, hypoxia and soluble agonists. While, larger microvesicles (MVs) (100 nm–1 μm) and apoptotic bodies (1–5 μm) directly bud outward from plasma membrane [29]. Adenosine diphosphate (ADP)-ribosylation factor 6 stimulates phospholipase D activity, which in turn facilitates extracellular signal-regulated kinase activation, which phosphorylate myosin light chain kinase 2 (which contracts cytoskeleton), causing stimulation of serine phosphorylation of myosin II that ultimately triggers release of MVs. As EVs are very heterogeneous in size and content, and due to a lack of reliable tools and specific markers to distinguish EVs subtypes, good classification of exosomes and MVs is an ongoing challenge.

But Mesenchymal stem cell microvesicles (MSC-MVs) are identified as the best characterized according to guidelines of the Minimal Information for Studies of MVs [30]. Previous studies have demonstrated the role of extracellular vesicles in sperm maturation, motility as well as its membrane integrity, and function [31]. Also, H. Liu et al. [32] showed that stem cell derived extracellular vesicles can alleviate testicular torsion-detorsion injury by reducing oxidative stress and inhibit inflammation and promote the proliferation and migration of spermatogenic cells. The reports on beneficial effects of MSC- MVs in inflammation and tissue repair have triggered a significant interest into the application of MSC- MVs as a cell-free therapy. As MSC-MVs -based therapy has several advantages over cellular therapies. Additionally, it was found that MVs have strong potential as a cell-free treatment for tissue regeneration because they can retain the therapeutic effects of their parent cells and do not have the safety concerns associated with cell therapy [33]. In addition, MVs can be promising in the treatment of neural diseases to rescue the degenerating cells and they are very important for the immune system, the cardiovascular system and act as anti-inflammatory, anti-apoptotic and proangiogenic. Also, MSC- MVs exert beneficial effects in skin wound healing, kidney injury, graft versus host disease, stroke and sepsis [34].

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