

Vitamin C and oxidative stress in the seminiferous epithelium

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ABSTRACT

In this article, we focus on the fundamental role of vitamin C transporters for the normal delivery of vitamin C to germ cells in the adluminal compartment of seminiferous tubules. We argue that the redox status within spermatozoa or in semen is partly responsible for the etiology of infertility. In this context, antioxidant defence plays a critical role in male fertility. Vitamin C, a micronutrient required for a wide variety of metabolic functions, has long been associated with male reproduction. Two systems for vitamin C transport have been described in mammals. Facilitative hexose transporters (GLUTs), with 14 known isoforms to date, GLUT1-GLUT14, transport the oxidized form of vitamin C (dehydroascorbic acid) into the cells. Sodium ascorbic acid co-transporters (SVCTs), SVCT1 and SVCT2 transport the reduced form of vitamin C (ascorbic acid). Sertoli cells control germ cell proliferation and differentiation through cell-cell communication and form the blood-testis barrier. Because the blood-testis barrier limits direct access of molecules from the plasma into the adluminal compartment of the seminiferous tubule, one important question is the method by which germ cells obtain vitamin C. Some interesting results have thrown light on this matter. Expression of SVCT2 and some isoforms of GLUT transporters in the testis have previously been described. Our group has demonstrated that Sertoli cells express functionally active vitamin C transporters. Kinetic characteristics were described for both transport systems (SVCT and GLUT systems). Sertoli cells are able to transport both forms of vitamin C. These findings are extremely relevant, because Sertoli cells may control the amount of vitamin C in the adluminal compartment, as well as regulating the availability of this metabolite throughout spermatogenesis.

Key words: ascorbic acid, oxidative stress, GLUT, Sertoli cells, SVCT, vitamin C transporters

INTRODUCTION

The testis is a complex organ that performs two crucial functions: a) synthesis and secretion of testosterone and other hormones through the process of steroidogenesis; and b) production of a sufficient number of competent spermatozoa through the process of spermatogenesis to attain fertility (Amann & Schanbacher, 1983). Both processes are intimately coupled, in their essential requirement for adequate levels of testosterone for the normal production and maturation of spermatozoa. To accomplish this, the testis is organized into two main compartments: the tubular compartment and the interstitium. The seminiferous tubules are formed by Sertoli cells that provide structural support for the germ cells, and by the peritubular myoid cells that surround the tubules (Kerr et al., 1992; Gülkesen et al., 2002). The interstitium is composed of Leydig cells, macrophages, fibroblasts, and blood vessels. These structures are embedded in the extracellular matrix, which is located immediately adjacent to the seminiferous epithelium, between the myoid cells and the basal surfaces of Sertoli cells and spermatogonia, and acquires the specialized form of the basement membrane, which represents an important component of the interstitial tissue (Yazama et al., 1997). *In vitro* studies have shown that the extracellular matrix plays a crucial role in Sertoli cell function and

development (Dym, 1987; Skinner, 1989; Davis et al., 1990). Leydig cells produce testosterone, which is required for critical communication between Sertoli cells and developing germ cells (Prante et al., 2008). This close communication creates a very special microenvironment for differentiating germ cells, where Sertoli cells regulate the access of molecules to this space.

Compartmentalization and the blood-testis barrier

The seminiferous epithelium is divided functionally in two compartments, so-called basal and adluminal (Stechell, 1967; Dym & Fawcett, 1970; Setchell & Main, 1978). Electron opaque tracer studies have demonstrated that the basal compartment contains diploid cells, spermatogonia types A and B and preleptotene and leptotene spermatocytes which divide by mitosis. The adluminal compartment however comprises an isolated environment in which more differentiated spermatocytes (zygotene, pachytene, diplotene, secondary spermatocytes) undergo meiosis and spermatids differentiate into spermatozoa (Dym & Fawcett, 1970; Dym & Cavicchia, 1978). This division forms the blood-testis barrier. Throughout the mammalian spermatogenic pathway, differentiating spermatogenic cells are situated in close contact with somatic Sertoli cells, and

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their interaction has been considered to be essential for the proliferation, differentiation and survival of spermatogenic cells (Russell et al., 1990; Lui et al., 2003; Maeda et al., 2007). During spermatogenesis, a process by which a single spermatogonium develops into 256 spermatozoa (Mruk & Cheng, 2004), developing germ cells migrate progressively across the seminiferous epithelium from the basal to the adluminal compartment (Lie et al., 2008). Although Sertoli cells extend from the basal compartment into the adluminal compartment, the two tubular compartments are separated by junctional complexes (tight and adherens junctions) between neighbouring Sertoli cells, that function as the major component of the blood-testis barrier (Fawcett et al., 1970; Dym & Fawcett, 1971; Russell, 1977; Holash et al., 1993; Li et al., 2006). This barrier, dividing the seminiferous epithelium, selectively restricts the passage of many substances contained in the general circulation (Dym, 1973; Pelletier & Byers, 1992; Kato et al., 2005; Li et al., 2006). The integrity of the blood-testis barrier is a prerequisite for normal spermatogenesis. There is also some evidence that peritubular myoid cells and endothelial cells of testicular blood vessels contribute to barrier function (Plöen & Setchell, 1992; Holash et al., 1993). As well as tight junctions, gap junctions are also present in the blood-testis barrier as shown by ultrastructural analysis in rats (Dym & Fawcett, 1970; Gilula et al., 1976; Batias et al., 2000). The blood-testis barrier sequesters germ cells usually considered antigenic, protecting the differentiated germ cells from the autoimmune response (Pelletier & Byers, 1992; Willing et al., 1998; Hemendinger et al., 2002) and from exposure to many compounds found in blood or interstitial tissue fluid which can be harmful to germ cells (Waites & Glawell, 1982; Bart et al., 2004; Augustine et al., 2005). The unique components of the tubular fluid in the adluminal compartment formed by Sertoli-Sertoli tight junctions create the necessary chemical microenvironment for completion of meiosis and spermiogenesis (Fenton et al., 2002; Xia et al., 2006). A key feature of the blood-testis barrier is its selective permeability to solutes and regulation of the movement of small molecules (Meng et al., 2005). Membrane transporters at barrier level are thus essential for regulation of molecule traffic (Maeda et al., 2007). Several substances believed to play key roles in the development and maintenance of testicular function are secreted by Sertoli cells and concentrated in the isolated adluminal compartment of the testis.

The blood-testis barrier is formed at approximately 15-18 days of age in rat and 10-16 days postpartum in mouse, as constituted by Sertoli cell tight junctions located in the basal third of the seminiferous epithelium (Shinohara et al., 2003).

The existence of a blood-testis barrier formed by Sertoli-Sertoli tight junctions is well documented. Distention of seminiferous tubules, accumulation of fluid after ligation of the efferent ducts (Setchell, 1980) and the inability of large molecules such as lanthanum and peroxidase to penetrate the testis (Dym & Fawcett, 1970; Neaves, 1973), are properties that bear proof of the impermeable barrier residing in seminiferous tubules. There are also differences in composition between the blood plasma and testicular lymph, and between the blood plasma and fluid in the lumina of seminiferous tubules and rat testis. Isolated tubules have been used to study the rate at which substances enter the adluminal compartment

(Setchell & Singleton, 1971; Setchell & Waites, 1975). Inhibition of microfilaments by cytochalasin D results in alteration of the Sertoli cell barrier, as evidenced by greater entry of radiolabelled inulin into the lumina of seminiferous tubules (Weber et al., 1988). Also, a single intratesticular injection of dilute glycerol solution alters the permeability of the blood-testis barrier to macromolecules such as inulin and albumin (Eng et al., 1994).

The Sertoli cell

The seminiferous epithelium is mainly composed of Sertoli (sustentacular epithelial cells) and germ cells. The Sertoli cell is an irregularly-shaped, columnar cell extending from the base to the apex of the seminiferous tubules in rats, and occupies a volume of approximately 17-19% in the seminiferous epithelium of the adult rat. When Enrico Sertoli described these cells in 1865, he drew scientific attention because of their close structural relationship with spermatogenic cells. It is a currently well-known fact that Sertoli cells play a central role in fetal gonad development and postnatal spermatogenesis. Sertoli cells have been extremely difficult to study morphologically because they have a constantly changing, three-dimensional relationship with developing germ cells. Resting upon the lamina propria of the seminiferous tubule, Sertoli cells are the only somatic cells within the seminiferous tubules, and they provide the microenvironment required for germ cell development. Their cytoplasmic processes extend to the lumen of the seminiferous tubule and envelope the developing germ cells. They provide the only communication link across the blood-testis barrier. Structurally, a Sertoli cell junctional complex is composed of occluding, gap, close, and adhering junctions (Pelletier & Byers, 1992). These intercellular junctions play an essential role in spermatogenesis (Parvinen, 1982; Russell & Peterson, 1985).

Sertoli cells *in vivo* are highly polarized and interact with the inner (adluminal) and outer (basal) fluids (Janecki & Steinberger 1986). Because of the physical separation produced by tight junctions, germ cells have to move from one compartment to the other, thus defining the polarity of the epithelium (Maeda et al., 2007). This special environment is maintained by polarized transport and the secretory function of the Sertoli cells (Janecki et al., 1988).

The composition of the spermatogenic cell population in contact with each Sertoli cell is in constant flux, and Sertoli cell structure and gene expression varies with changes in the spermatogenic cell population at different moments of the seminiferous epithelial cycle. Therefore, many separate pathways of Sertoli-Sertoli and Sertoli-spermatogenic cell communication may be needed, and dynamic regulation of the individual pathways is likely. Paracrine, endocrine, and autocrine communication pathways play major roles in the function of the seminiferous epithelium (Russell et al., 1990; Prante et al., 2008). Gap junctions between the different cell types establish additional communication pathways that are, in turn, subject to regulation by paracrine and endocrine factors (Risley, 2002). In the testis within the seminiferous tubules, there is strong expression of the protein connexin 43 that correlates with the stages of spermatogenesis (Risley et al., 1992; Batias et al., 1999; Defamie et al., 2001). This protein also co-localizes with the gap junctions of the Sertoli cell junctional complex (Risley et al., 1992; Pelletier, 1995).

Sertoli cells are very important for proper development of germ cells. They perform numerous structural and supportive functions and play a central role in sex determination (Karl & Capel, 1998). They are physically reshaped by germ cells and possess many cytoplasmatic processes because each Sertoli cell is “nursing” about 30-50 germ cells at four or five different stages of their development at any given time during the epithelial cycle (Kelly et al., 1991; Risley et al., 2002). The germ cells are anchored by desmosome-like, ectoplasmic specializations within the Sertoli cells. Organelles within the Sertoli cells, including the endoplasmic reticulum, have an active role during nuclear elongation and acrosome formation (Amann & Schanbacher, 1983). In rat, Sertoli cells cease to proliferate at around day 20 postnatal and it is currently accepted that the number of Sertoli cells in the mature testis is established during puberty and that Sertoli cell number is constant in an adult male (Lino, 1971; Hochereau-de Reviers & Lincoln, 1978). In addition, the Sertoli cell number determines an upper limit for sperm production, a crucial detail (Sharpe, 1994, 1999; Buzzard et al., 2002). Sertoli cells facilitate the progression of degenerating germ cells and their remains (Griswold, 1995; Griswold, 1998; Buzzard et al., 2002; Mruk & Cheng, 2004; Oliveira et al., 2009) and it has been demonstrated that apoptotic spermatogenic cells and residual bodies are phagocytosed and degraded by Sertoli cells during mammalian spermatogenesis (Shiratsuchi et al., 1999; Xiong et al., 2008, 2009).

Other novel Sertoli cell features have also come to light. These cells provide localized immunoprotection to transplant tissue, protecting the grafts from rejection and supporting their use as a viable graft source, facilitating xenotransplantation in Parkinson’s disease (Sanberg et al., 1996, 1997; Willing et al., 1999). Both porcine and rat Sertoli cells have been used as graft facilitators (Shamekh et al., 2006).

Sertoli cells secrete numerous biological factors for germ cells, such as, nutrients, ions, hormones, carbohydrates and growth factors, and control the composition of seminiferous fluid (Wright et al., 1981; Griswold 1995; Eskild et al., 2000; Mruk & Cheng, 2004). In response to follicle stimulating hormone, FSH (Tres & Kierszenbaum, 1983; Chaudhary et al., 1996), and in the presence of testosterone, Sertoli cells secrete fluid and specific products such as androgen-binding protein and inhibin (Sanborn et al., 1981; Steinberger, 1981).

In mammals, spermatogenesis occurs under the influence of Sertoli cells, which control the process through receptor-mediated cell-cell contacts and via molecules secreted by Sertoli cells into the adluminal compartment in the seminiferous tubules (Jegou, 1993). Numerous experiments have utilized co-cultures of Sertoli cells and germ cells to investigate the way in which Sertoli cell factors influence germ cell development. Sertoli cells stimulate germ cell RNA and DNA synthesis, inducing the appearance of germ cell surface antigens, and maintaining spermatogenic cell glutathione synthesis (Syed et al., 1999). Moreover, Sertoli cells are necessary for provision of adequate lactate levels for germ cell development (Galardo et al., 2008).

Thus, selective movement of compounds across Sertoli cells compromises the physiological or functional aspect of the blood-testis barrier. Because of their vital role in regulating the exchange of materials between blood and adluminal compartment, thereby protecting the developing germ cells from toxic insults, Sertoli cells are a toxicologically important part of the male reproductive system.

Infertility, oxidative stress and vitamin C.

Vitamin C (L-ascorbic acid, ascorbate) has long been associated with fertility. This vitamin is an essential water-soluble micronutrient required for an array of biological functions. It was first isolated in 1928, by the Hungarian biochemist and Nobel Prize winner Szent-Györgyi. It is unstable, easily oxidized acid and can be destroyed by oxygen, alkali and high temperature (Iqbal et al., 2004).

Recent data suggest that infertility is a major clinical problem and that it occurs in one in four couples (Guillette et al., 1994; Auger et al., 2001; Arabi et al., 2004). Sperm counts in healthy men around the world have fallen by about 50 per cent in the last 50 years in the Western world (Auger et al., 1995; Kasahara et al., 2002). This may be attributed to the insufficient spermatozoa (oligozoospermia) or the lack of spermatozoa (azoospermia) (Yazama et al., 2006). Carlsen et al., (1992) reported in declining sperm concentrations in normal men (Irvine et al., 1997). Infertility may also be due to damaged somatic Sertoli cells. In male heavy smokers, Sertoli cell functions deteriorate (Pflieger-Bruss, 2004). Moreover, some toxicants impair male fertility target Sertoli cells, illustrating the importance of these cells in human male reproduction (Monsees et al., 1999). In recent years, the deterioration in human fertility potential has been partly due to effects of increasing exposure to toxic chemicals such as pesticides (Monsees et al., 2000; Defamie et al., 2001), cigarette smoke and nicotine (Arabi et al., 2004; Pflieger-Bruss et al., 2004). Dietary deficiencies have also been linked to sperm oxidative damage by several research groups. Fifteen percent of all couples in the US are infertile. The male factor is responsible for 25 % of these cases (Sharlip et al., 2002; Liu et al., 2006; Agarwal et al., 2008). One male infertility mechanism is the excess production of reactive oxygen species (ROS) in sperm (Sharma & Agarwal, 1996; Chen et al., 2001; Kasahara et al., 2002; Agarwal et al., 2008). This induces nuclear DNA fragmentation, lipid peroxidation and protein cross links and results in cell death (Alvarez et al., 1987; Steenken, 1989; Tramer et al., 1998; Liu et al., 2006). In semen, production of free radicals comes from two main sources; leukocytes and sperm (Tremellen et al., 2008). A spermatozoa, like any other cell, is constantly facing the “oxygen-paradox” (Sies, 1993). Just as oxygen is essential for sustaining life, physiological levels of ROS are necessary for maintenance of normal cell function (Fraczek & Kurpisz, 2005). For spermatozoa, small amounts of ROS are necessary for stimulation of the tyrosine phosphorylation associated with sperm capacitation (Gagnon et al., 1991; Aitken 1997; Mruk, 1998; Baker & Aitken, 2005). However, ROS has also been shown to be detrimental to cell function and survival (de Lamirande et al., 1995). Mazilli et al., (1994) found significantly elevated levels of superoxide anion in 87 % of infertile patients, although hydrogen peroxide is the major ROS produced in sperm. The respiratory chain of the mitochondrion is one of the most productive ROS generating systems in sperm. Spermatozoa are particularly sensitive to ROS assault because of the existence of high quantities of polyunsaturated fatty acids in their own membranes and the lack of capacity for DNA repair (Aitken & Clarkson, 1987; Álvarez & Storey, 1995; Kim & Parthasarathy, 1998; Donnelly et al., 2000). Increased ROS levels have been correlated with decreased sperm motility (Álvarez & Story, 1982; Aitken et al., 1989; Iwasaki & Gagnon, 1992; Jedlinska-Krakowska et al., 2006), retention of excess residual

cytoplasm (Baker & Aitken, 2005), midpiece abnormalities (Rao et al., 1989), abnormal morphology (Aitken et al., 1994), DNA damage in the male germ line cells that is associated with mutation, poor fertilization rates following *in vitro* fertilization, defective pre-implantation embryonic development and high rates of miscarriage in the offspring, and finally, increased incidence of birth defects and genetic diseases (Álvarez & Storey, 1982; Fraga et al., 1991; Aitken et al., 1998; Lim et al., 1998; Agarwal et al., 2008). These problems, associated with sperm DNA damage, result in defective spermatogenesis (Agarwal et al., 2008). A significant positive association has been reported between active smoking (Wong et al., 2000) and sperm DNA fragmentation (Sun et al., 1992), axonemal damage (Zavos et al., 1998) and decreased sperm count (Vine et al., 1996). Smoking results in a 48 % increase in seminal leukocyte concentrations and 107 % increase in seminal ROS levels (Aitken & West, 1990). Smokers have decreased levels of seminal plasma antioxidants such as vitamin C (Tremellen et al., 2008). Spermatozoa from subjects who smoked are significantly more sensitive to acid-induced DNA denaturation than non-smokers (Potts et al., 1999). In a study of 655 smokers and 1131 non-smokers, it was shown that cigarette smoking was associated with a significant decrease in sperm density, total sperm count, and total number of motile sperm (Kunzle et al., 2003). But, Tremellen et al., (2008) observed a significant correlation between vitamin C intake and sperm concentration. The non-enzymatic antioxidants present within semen include ascorbic acid.

Studies have shown that antioxidants protect spermatozoa from ROS-induced production of abnormal spermatozoa, they scavenge ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality in smokers, reduce cryodamage to spermatozoa, block premature sperm maturation and stimulate spermatozoa (Fraga et al., 1991). Seminal fluid contains antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase/glutathione reductase in addition to non-enzymatic antioxidants such as vitamin C, urate, vitamin E, pyruvate, glutathione, albumin, vitamin A, ubiquinol, taurine, and hypotaurine (Kim & Parthasarathy, 1998). Supplementation of sperm preparation clinical media with antioxidant has often been related as a way of reducing ROS-induced damage in sperm (Donnelly et al., 2000).

Vitamin C is considered a major antioxidant in the testis (Augustine et al., 2005). It neutralizes ROS and prevents sperm agglutination (Dawson et al., 1992; Aitken 1999). It is a donor of electrons for redox systems, prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by hydrogen peroxide radical (Jedlinska-Krakowska et al., 2006). Administration of 200 mg of vitamin C orally, together with vitamin E and glutathione, for a period of 2 months, significantly reduced hydroxyguanine (8-OH-dG) levels in spermatozoa and also increased the sperm count (Kodama et al., 1997). Daily oral supplementation of vitamin C and E for 2 months reduced the number of TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) positive spermatozoa (Greco et al. 2005a). Moreover, a marked improvement in clinical pregnancy and implantation rates has been reported after antioxidant treatment. Addition of vitamin C and E to the sperm preparation media during density gradient separation protected sperm from DNA damage (Hughes et al., 1998; Greco et al., 2005b; Agarwal et

al., 2008). In men who had decreased sperm count, motility or and increased abnormal sperm count, the vitamin C level was found to be decreased. High vitamin C concentration caused an increase in the epididymal sperm concentration and serum testosterone level of rats. So, vitamin C supplementation seems to improve semen quality (Sönmez et al., 2005)

Gentamycin induces oxidative damage by increasing free radical formation, lipid peroxidation and by decreasing antioxidant levels. These effects were reversed following vitamin C supplementation, implying a direct role for this vitamin in protection against oxidative damage (Narayana, 2008). Also, some toxins as cisplatin, that decrease vitamin C levels, induce an increase of ROS and damage germ cells, with a significant decline in sperm count (Narayana, 2008) and a higher incidence of DNA damage (Thiele et al., 1995). Vitamin C significantly, but not completely, protects against cisplatin-induced testicular damage (Narayana et al., 2009). Levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine, a major product of oxidative damage, were measured in DNA isolated from human sperm. Results showed that high levels of this product was correlated with low seminal plasma vitamin C levels (Fraga et al., 1991). Since the 1920s, dietary vitamin C has been known to play a critical role in protecting male germ cells against oxidative damage. A vitamin C deficient diet causes massive degeneration of the seminiferous epithelium with desquamation into the lumen of the testis tubules in guinea pig (Lindsay & Medes, 1926). Vitamin C is present at a high concentration in seminal fluid as compared to plasma (400 vs 60 mM), but present in low levels in the infertile seminal plasma (Lewis et al., 1997). The amount of testicular vitamin C declines significantly after treatment with toxic molecules such as chrome and cadmium. Although ascorbic acid content in the testis remains unaltered during development, an ascorbic acid deficiency disrupted spermatogenesis in the testis of chrome-exposed animals, accounting for the drastic fall in sperm count (Augustine et al., 2005) and markedly increased rates of sperm abnormality (Achayra et al., 2006). However, vitamin C and vitamin E supplements given to cadmium-induced mice groups reduced lipid peroxidation and increased sperm count (Achayra et al., 2008).

Germ cell detachment has been shown to occur in mixed cultures of Sertoli and germ cells in response to some known *in vivo* testicular toxins. Such cultures were also treated with the oxygen radical generating system of xanthine/xanthine oxidase (Anderson & Francis, 1993). There was an increase in germ cell detachment with this treatment that was reduced by vitamin C but not by vitamin E at the doses administered. Together, these results suggest that vitamin C supplementation could protect reproductive somatic cells against toxins that act through oxygen radical mechanisms (Anderson & Francis, 1993).

Other studies of the relation between vitamin C and germ cell development have shown that germ cell differentiation in organ culture of testis from infant rats was improved with serum and vitamins A, C and E (Steinberger & Steinberger, 1966). Chinoy et al. (1986), demonstrated that vitamin C was essential for maintaining the physiological integrity of the androgen target reproductive organs. Because vitamin C is an important cofactor for hydroxylation of collagen (an important component of the extracellular matrix), malfunction of the cell-cell interaction of the testicular cell relates to abnormal spermatogenesis and infertility problems (Yazama et al.,

2006). Sapra et al., (1987) observed that vitamin C deficiency in guinea pigs caused a decrease in weight of reproductive organs. Other studies indicated that the weight of the testis and accessory sex organs were significantly decreased by some toxic substances, and that administration of vitamin C reversed this reduction (El-Missiry et al., 1999; Das et al., 2002).

Vitamin C and vitamin C transporters

Vitamin C synthesis occurs in the liver and the body requires this six-carbon keto-lactone for normal physiological functions. However, unlike most animals, humans are unable to synthesize vitamin C, making it necessary to include it in the diet, in foods, or as a supplement (Subramanian et al., 2008). This inability to synthesize ascorbic acid from glucose, is due to a lack of the active enzyme, L-gulonolactone oxidase (Burns, 1959, Nandi et al., 1997; Linster & Van Schaftingen, 2007). Vitamin C acts as a cofactor for several important enzymes. It assists the metabolism of tyrosine, folic acid, and tryptophan, and helps to lower blood cholesterol. It also contributes to the synthesis of carnitine, collagen and catecholamines that regulate the nervous system (Kubler and Gehler, 1970; Iqbal et al., 2004). It is needed for tissue growth and wound healing (Kallner et al., 1979), assists the formation of neurotransmitters and increases the absorption of iron. As an antioxidant, it protects the body from the harmful effects of free radicals and pollutants (Brzozowski et al., 2001; Guaiquil et al., 2001). It functions as an essential independent cellular antioxidant even in the presence of a vast excess of glutathione under oxidative challenge (Montecinos et al., 2007). It is a very potent scavenger of superoxide and other reactive oxygen species (Englard & Seifter, 1986). Of the two chemically distinct forms of vitamin C, only the reduced form, ascorbic acid, is present in human plasma, cells and tissues, and the cellular concentration of this vitamin can exceed the plasma level (60 mM) by several orders of magnitude (Guaiquil et al., 1997). Ascorbic acid is concentrated in the testis at a concentration of 200 mM (Moser, 1987). Sertoli cell tight junctions form the blood-testis barrier to protect developing spermatogenic cells against harmful agents and to provide nutrients from the blood stream for spermatogenesis. Accordingly, Sertoli cells should regulate the movement of various nutritious and xenobiotic compounds via selective membrane transporters. However, information on such membrane transporters is limited (Maeda et al., 2007) and there is no information regarding the mechanism of vitamin C transport through the blood-testis barrier. Undoubtedly, this vitamin plays an essential role in male reproduction. The method by which ascorbic acid enters the adluminal compartment to reach the developing germinal cells is a problem that has been unsolved for many years.

Vitamin C is accumulated within cells by two distinct mechanisms involving transport of both reduced and oxidized forms (Spielholz et al., 1997; Erichsen et al., 2001). In the first mechanism, vitamin C (ascorbic acid) is the specific substrate transported, by a high-affinity, low-capacity system, that is sodium- and temperature-dependent (Daruwala et al., 1999; Tsukaguchi et al., 1999; Liang et al., 2002; Godoy et al., 2007). The molecular identity of this system has been delineated following cloning of two transporters (from human kidney cDNA library): the human sodium vitamin C cotransporters, SVCT1 and SVCT2 (products of the SLC23A2 and SLC23A1 genes, respectively; Daruwala et al., 1999). SVCT1 has a 1797

bp open reading frame encoding a 598 amino acid polypeptide. The 1953 bp open reading frame of SVCT2 encodes a 650 amino acid polypeptide (Wang et al., 2000). Both transporters mediate ascorbic acid uptake when expressed in a variety of cell systems including small intestine, smooth muscle, epithelial and endothelial cells (Tsukaguchi et al., 1999). In a *Xenopus laevis* oocyte expression system, both transporters were functionally expressed (Wang et al., 1999, 2000) and did not transport dehydroascorbic acid (Daruwala et al., 1999). SVCT1 has an apparent transport Km in the range of 65-237 mM, and is electrogenic. SVCT2 has an apparent Km of 8-62 mM (Wang et al., 2000; Savini et al., 2007; Steiling et al., 2007). SVCT2 also requires calcium and magnesium to function (Godoy et al., 2007). Hydropathy plot analyses predict that these two proteins have a 12 transmembrane spanning topology with cytoplasmic C and N terminal domains as well as multiple consensus sites for glycosylation and phosphorylation. SVCT1 and SVCT2 transporters share a 66 % amino acid homology (Boyer et al., 2005), although they are sorted into distinct membrane compartments in epithelial cells. Recently, the expression of SVCT transporters has been described in many cell types and transport results show that ascorbic acid uptake maintains the same kinetic characteristics (Maulén et al., 2003; Castro et al., 2007; 2008).

In the second mechanism, oxidized vitamin C (dehydroascorbic acid) is transported by facilitative hexose transporters, via a low-affinity, high-capacity transport system that is sodium-independent. Once transported into the cell, dehydroascorbic acid is immediately reduced to ascorbic acid allowing for recycling of ascorbate (Guaiquil et al., 1997; May, 1998). The first identified protein of the GLUT family multimembrane spanning facilitative transporters, GLUT1 is a type 2 integral membrane protein composed of 12 transmembrane domains that delineate six extracellular loops (Mueckler et al., 1985; Montel-Hagen et al., 2008). GLUT1 has been described as the main functional transporter of glucose in most transformed cells as well as in various hematopoietic cell lineages (Mueckler, 1994, 1995; Hruz & Mueckler, 2001). Fourteen GLUT isoforms have now been identified in the human genome. GLUT1 transports L-dehydroascorbic acid, an oxidized intermediate of ascorbic acid (Vera et al., 1993; Vera et al., 1995; Rumsey et al., 1997). Dehydroascorbic acid entry via GLUT family transporters was initially investigated because of the structural similarities between dehydroascorbic acid and glucose. All mammalian cells contain one or more members of this GLUT protein family (Joost & Thorens, 2001). The transporters, present in all cells and tissues (Concha et al., 1997), have a high degree of stereoselectivity allowing for bidirectional transport of substrate with passive diffusion down its concentration gradient (Wilson, 2005).

Evidence obtained from expression studies in *Xenopus laevis* oocytes indicates that GLUT1, GLUT2, GLUT3, and GLUT4 are dehydroascorbic acid transporters (Vera et al., 1993; Vera et al., 1995; Rumsey et al., 1997). Transport of dehydroascorbic acid via glucose transporters is kinetically and biologically separable from reduction and accumulation of this substrate (Vera et al., 1995; Rivas et al., 1997).

Vitamin C transporters have been found to be abundant in cells forming a barrier (Hussar et al., 2002). SVCT2 and GLUT1 are expressed in the blood-retinal barrier. Dehydroascorbic acid crosses the blood-brain barrier through GLUT1 (Agus et al., 1997) and ascorbic acid through SVCT2. The polarization of

the expression of vitamin C transporters has been extensively reported in physiological barriers. For example, SVCT1 is expressed in the apical membrane of polarized MDCK cells (Varma et al., 2009). SVCT1 is also selectively sorted to the apical membrane compartment in Caco-2 cells (Maulén et al., 2003), allowing vectorial uptake of ascorbic acid in differentiated cells. These results have a direct impact on understanding the mechanism of vitamin C transport across the intestinal barrier.

Transporters of vitamin C in testicular epithelium

GLUT transporter expression has been related to fertility. Male diabetes may cause male infertility by altering steroidogenesis, sperm motility and levels of GLUT protein expression (Kim & Moley, 2008).

Different members of the GLUT protein family are expressed in testis (Carosa et al., 2005). Expression of GLUT1, GLUT2, GLUT3, GLUT5 and GLUT8 have been demonstrated in rat, mouse and human testis (Burant et al., 1992; Ulisse et al., 1992; Burant & Davison, 1994; Angulo et al., 1998; Doege et al., 2000; Ibberson et al., 2000; Carosa et al., 2005; Galardo et al., 2007; Angulo et al., 2008; Table 1).

GLUT3 is highly expressed in rat, mouse, human and bovine sperm (Angulo et al., 1998; Simpson et al., 2008). GLUT1, GLUT2 and GLUT3 are expressed in early spermatocytes, peritubular myoid cells, macrophage-like interstitial cells and testicular endothelial cells (Rauch et al., 2006; Kokk et al., 2007; Table 1) and spermatids express GLUT1, GLUT2 and GLUT3 (Rauch et al., 2006; Kokk et al., 2007).

GLUT2 and GLUT3 have been identified in Sertoli cells of adult rats (Burant & Davison, 1994; Kokk et al., 2007). Galardo et al., (2008) demonstrated the expression of GLUT1 and GLUT3 glucose transporters in testicular tissue sections and Sertoli cell primary culture. GLUT1 in particular, is expressed in both adult and immature rat Sertoli cells (Galardo et al., 2008; Table 1).

Ulisse et al., (1992) showed that Sertoli cells transport glucose. Hall & Mita (1984) revealed that Sertoli cells transport 3-o-methyl-D-(14C) glucose and that this transport is competitively inhibited by glucose. These authors described glucose transport capacity in Sertoli cells, and this transport was kinetically related to GLUT transporter activity (Table 1). However, only SVCT2 expression has been described in testis, specifically in interstitial cells and spermatocytes (Tsukaguchi et al., 1999; Wang et al., 2000; Table 2).

We studied the expression of SVCT transporters in rat testis. SVCT1 and SVCT2 are expressed in spermatogenic cells and Sertoli cells in the seminiferous epithelium (Fig.1; Table 2) and in isolated rat spermatocytes and spermatids (unpublished data; Table 2). In testis, SVCT1 and SVCT2 was observed at the basal and luminal surfaces of the seminiferous tubules (Fig. 1A, B, C, D). Intense co-localization between WT-1 (Sertoli cell marker; transcription factor present in Wilm's tumor) and SVCT2 was observed at the basal region of the tubules (Fig. 1D). Immunofluorescence and Western blot analysis demonstrated that mouse, bovine and human spermatozoa expressed SVCT1 and SVCT2 proteins (Fig 2; Table 2). Therefore, spermatogenic cells express both vitamin C transport systems, allowing these cells to take up ascorbic acid and dehydroascorbic acid under different conditions.

Culturing Sertoli cells has become an increasingly popular means by which isolated Sertoli cells may be studied (Kodani & Kodani, 1996; Steinberger et al., 1975; Dym et al., 1987; Sanborn et al., 1987; Janecki and Steinbergergerger 1988). To study the molecular and functional characterization of vitamin C transporters in the blood-testis barrier, we have used the 42GPA9 cell line from mouse Sertoli cells (Angulo et al., 2008). This cell line, in addition to expressing specific markers for Sertoli cells (Bourdon et al., 1998; Angulo et al., 2008), has the ability to form junctional complexes between cells. These complexes comprise tight and gap junctions that mimic the blood-testis barrier (Lablack et al., 1998). This system was a useful model for the investigation of the way in which vitamin C may be transported to the adluminal compartment. We showed that Sertoli cells express both types of vitamin C

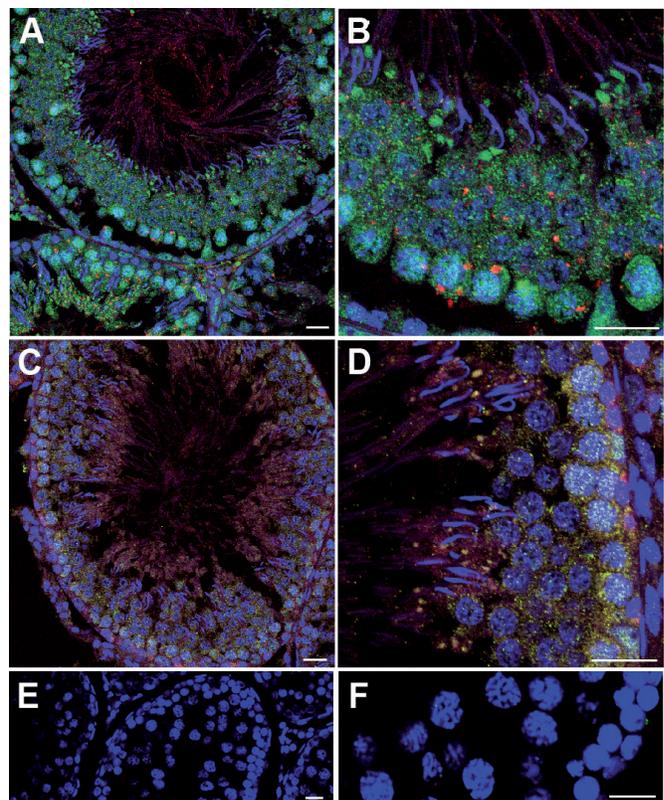


Figure 1. Ascorbic acid transporters are expressed in adult testis. Double-label immunofluorescence analysis using confocal microscopy, tissue sections from adult rat testis were incubated with SVCT1 (D-19; in red) an antibody directed against an appropriate N-terminus peptide of the rat protein (1: 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and WT-1 (Sertoli cell marker; in green) (C-19) an antibody against the C-terminus of the human protein (1: 50; Santa Cruz Biotechnology, Santa Cruz, CA, USA; **A** and **B**), or SVCT2 (G-19; in red) an antibody directed against an appropriate extracellular domain of the rat protein (1: 100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and WT-1 (C-19) (**C** and **D**), the slides were washed and incubated with anti-goat IgG-Alexa Fluor 594 (1:300; Invitrogen, Carlsbad, CA, USA) and anti-rabbit IgG-Alexa Fluor 488 (1:300; Invitrogen, Carlsbad, CA, USA). Nuclei were stained with TO-PRO 3 iodide (642/661; in blue). Incubation without first antibody was used as negative control (**E** and **F**). Scale bars are 20 μm.

Table 1**Dehydroascorbic acid transporter (GLUT) expression in testis**

Transporter	Tissue/ cel type expression	Function	Reference
GLUT1	Testis, Sertoli cells, spermatocytes, spermatids, spermatozoa, peritubular myoid cells, testicular endothelial cells, macrophage-like interstitial cells	Transport of glucose (3-methylglucose and 2-deoxyglucose) and dehydroascorbic acid in Sertoli cells, transport of glucose (2-deoxyglucose) in pachytene spermatocytes and round spermatids, transport of glucose (2-deoxyglucose) and dehydroascorbic acid in spermatozoa	Angulo et al., 1998; Riera et al., 2002; Kokk et al., 2004, 2007; Carosa et al., 2005; Rauch et al., 2006; Angulo et al., 2008; Galardo et al., 2008
GLUT2	Testis, Sertoli cells, spermatocytes, spermatids, spermatozoa, peritubular myoid cells, testicular endothelial cells, macrophage-like interstitial cells	Transport of glucose (3-methylglucose) and dehydroascorbic acid in spermatozoa	Angulo et al., 1998; Kokk et al., 2004, 2007; Angulo et al., 2008
GLUT3	Testis, Sertoli cells, spermatocytes, spermatids, spermatozoa, peritubular myoid cells, testicular endothelial cells, macrophage-like interstitial cells	Transport of glucose (3-methylglucose) and dehydroascorbic acid in Sertoli cells, transport of glucose (2-deoxyglucose) in pachytene spermatocytes and round spermatids, transport of glucose (2-deoxyglucose) and dehydroascorbic acid in spermatozoa	Haber et al., 1993; Burant & Davidson 1994; Younes et al., 1997; Angulo et al., 1998; Kokk et al., 2004, 2007; Rauch et al., 2006; Angulo et al., 2008; Galardo et al., 2008
GLUT4	Testis, Sertoli cells	undescribed	Angulo et al., 1998; Angulo et al., 2008

Table 2**Ascorbic acid transporter (SVCT) expression in testis**

Transporter	Tissue/ cell type expression	Function	Reference
SVCT1	Testis, Sertoli cells, spermatocytes, spermatids, spermatozoa	Transport of ascorbic acid in Sertoli cells	Angulo et al., 2008
SVCT2	Testis, Sertoli cells, spermatocytes, spermatids, spermatozoa	Transport of ascorbic acid in Sertoli cells	Tsukaguchi et al., 1999; Wang et al., 2000; Angulo et al., 2008

transport systems (Angulo et al., 2008). Prior to this finding, SVCT transporters had been poorly studied in testicular tissue. SVCT1 and SVCT2 expression was demonstrated by RT-PCR assays, immunofluorescence and Western blot (Angulo et al., 2008; Table 2). Transport results were consistent with the expression of more than one ascorbic acid transporter, with Km values for ascorbic acid transport similar to those described for SVCT transporters (Angulo et al., 2008). Ascorbic acid transporters were sodium-activated and specifically inhibited as for SVCT transporters. In addition, Sertoli cells transport oxidized vitamin C via two transport systems with distinct kinetic properties. Apparent Km values for dehydroascorbic acid confirmed the presence of at least two GLUT isoforms. Taken together, transport data and immunolocalization showed that GLUT1 and GLUT3 could be the most important dehydroascorbic acid transporters present in Sertoli cells (Angulo et al., 2008). These findings suggest an important role for vitamin C in the normal metabolism of this essential micronutrient, known to be required for the maintenance of spermatogenesis.

The expression of different isoforms of hexose transporters (GLUT) has been largely visualized as an adaptation to the requirement of different substrates for cell metabolism. The expression of many functionally active vitamin C transporters in spermatogenic cells suggests that adaptation to the seminiferous tubule, seminal fluid and oviduct fluid environment may be the reason for such diverse expression of vitamin C transporters.

Considering the findings, we can postulate a model for vitamin C metabolism in Sertoli cells that relates to delivery of vitamin C to developing spermatogenic cells. Ascorbic acid may enter the Sertoli cells through SVCT1 or SVCT2. Within the cell, it may be stored for use under different conditions or may pass across the Sertoli cell membrane to be delivered to spermatogenic cells in the adluminal compartment. For to occur, ascorbic acid must exit Sertoli cells. In some cell systems (erythrocytes, endothelial cells, and hepatocyte-like HepG cells) ascorbate efflux has been observed (Wilson, 2005). It is not known if a single transporter exchanges extracellular dehydroascorbic acid for intracellular ascorbate or if different transporters

mediate uptake and efflux (Wilson, 2005). In our model (Fig. 3), ascorbic acid can diffuse from the cytoplasm of Sertoli cells to the adluminal fluid through volume sensitive anion channels or by exocytosis of ascorbate in secretory vesicles. In this context, ascorbic acid reaches the adluminal fluid to maintain the concentration of the same and to be transported within the spermatogenic cells. Connexin proteins, which aggregate to form gap junctions and hemichannels in plasma membranes, may increase membrane permeability to ascorbate. Therefore, another possible ascorbic acid efflux may occur through gap junctions present in Sertoli cells, which connect with spermatogenic cells at different stages of development, thus delivering ascorbic acid directly to their cytoplasm (Fig. 3). Moreover, dehydroascorbic acid produced locally can enter Sertoli cells via GLUT

transporters. Inside these cells, this will be reduced to ascorbic acid by the local recycling enzymes. Dehydroascorbic acid could then be transported by GLUT transporters to the adluminal fluid where it may be taken up by spermatogenic cells via GLUT transporters (Fig. 3).

CONCLUDING REMARKS

Human male fertility is a complex process. Lifestyle factors, as well as various environmental agents, may impair male fertility (Pflieger-Bruss et al., 2004). The testis is sensitive to a variety of stressors, such as hyperthermia, inflammation, radiation and exposure to agents that induce apoptosis of germ cells. Because oxidative stress in the testis is one of the major factors that induces germ cell apoptosis, this organ has high concentrations of antioxidants such as ascorbic acid, to protect germ cells against oxidative damage (Luck, 1995). Ascorbic acid deficiency actually causes disturbance of spermatogenesis; thus the defence mechanism against oxidative stress plays a critical role in the maintenance of spermatogenesis. In the mammalian testis, Sertoli cells are strategically located to create a specific environment that promotes germ cell differentiation, and controls the entry of nutrients, hormones and other chemicals into the tubules of the testis, rendering the adluminal compartment an immuno-privileged site. As has been described, normal physiological functions of male reproductive tissues are maintained by the selective transfer of physiological and xenobiotic compounds across the blood-testis barrier, with

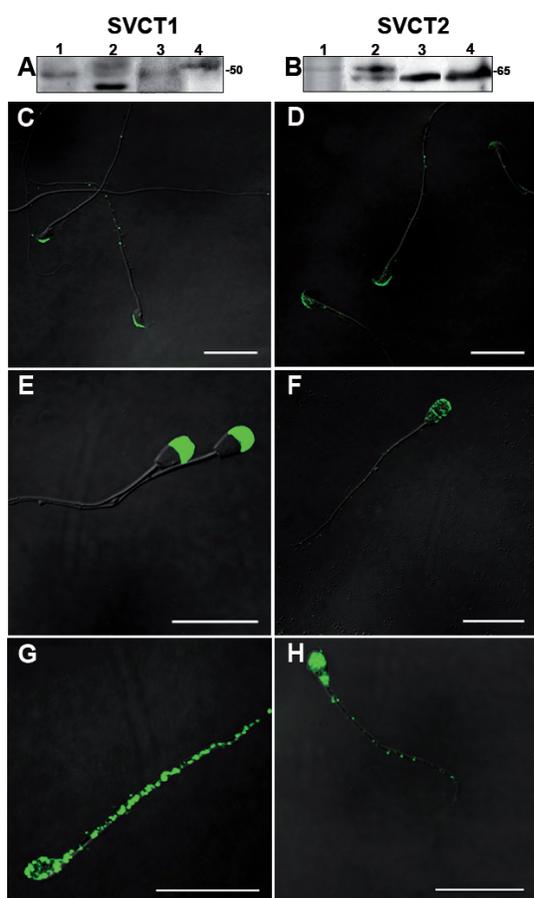


Figure 2. Ascorbic acid transporters, SVCT1 and SVCT2, are expressed in mammalian spermatozoa. A. Western blot analysis for SVCT1 using SVCT1 (D-19) antibody, and SVCT2 using SVCT2 (G-19). Lane 1: proteins isolated from mouse spermatozoa, lane 2: proteins isolated from bovine spermatozoa, lane 3: proteins isolated from human spermatozoa, lane 4: positive control using proteins obtained from rat liver (SVCT1) and rat brain (SVCT2). The numbers to the right indicate kDa. Immunofluorescence analysis for SVCT1 in mouse spermatozoa (C), bovine spermatozoa (E), human spermatozoa (G), and for SVCT2 in mouse spermatozoa (D), bull spermatozoa (F), human spermatozoa (H) using the same antibodies and a secondary anti-goat IgG Alexa Fluor 488. The images correspond to the superposition between confocal transmission and immunofluorescence images. Scale bars are 20 μ m.

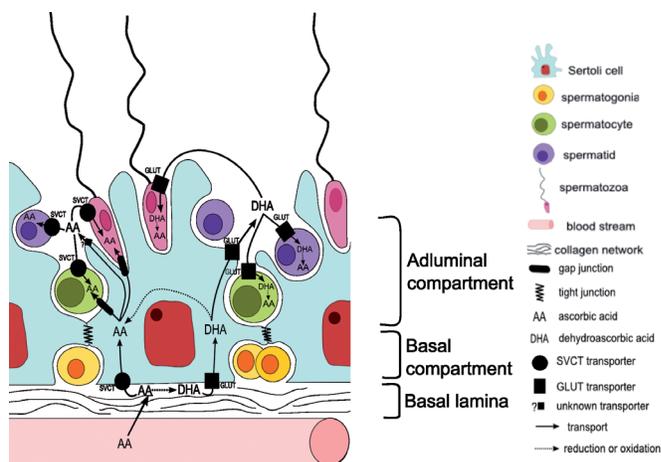


Figure 3. Vitamin C transport in seminiferous epithelium. Schematic representation of Sertoli cell interactions with germ cells and the role of these cells in vitamin C availability at the adluminal compartment. Vitamin C is present in the plasma mainly as ascorbic acid. Ascorbic acid transporters (SVCT, ●) and dehydroascorbic acid transporters (GLUT, ■). Ascorbic acid (AA) enters Sertoli cells via SVCT transporters and may then be transported to the adluminal space by an unknown transporter or pass through gap junctions (●) that allow for communication with developing germ cells. It is also possible that vitamin C enters (→) Sertoli cells as dehydroascorbic acid by GLUT transporters, and is rapidly reduced to ascorbic acid (---→) within the cell. Once transported into the cells, dehydroascorbic acid could then be directly transported by GLUT transporters to the adluminal fluid. All these possibilities should be considered in an attempt to understand vitamin C metabolism in the seminiferous epithelium.

part of this transfer being ascribed to membrane transporters. It was speculated by Augustine et al., (2005), that vitamin C must pass through Sertoli cells by either passive diffusion or facilitative transport to reach the adluminal space. In 2008, we clarified this issue by showing that Sertoli cells express both of two functionally active vitamin C transporters, giving these cells the capacity to metabolize vitamin C and regulate the delivery of this molecule to the germ cells in the adluminal compartment. That Sertoli cells are responsible for controlling vitamin C content in the adluminal compartment, is a new finding consistent with the concept that Sertoli cells provide a very particular microenvironment for the normal development of spermatogenesis.

In conclusion, SVCT and GLUT systems present in Sertoli cells specifically transfer ascorbic and dehydroascorbic acid transport across the blood-testis barrier. Ascorbic acid protects germinal cells from oxidative stress throughout spermatogenesis, preventing germinal cell damage and infertility.

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