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Electron Microscopic Study on the Effects of Melatonin on Early Spermatids in the Rat Testis

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Abstract: Melatonin is a neuroendocrine hormone secreted principally at night by pineal gland and regulates biological rhythms. Exogenous melatonin is used as a potential treatment for insomnia, sleep disorders and depression. The present investigation was undertaken to characterize the morphological defects in spermatids during early phases of spermiogenesis in melatonin-treated rats using electron microscopy. A single melatonin dose of either 0.05 or 0.1 mg kg^{-1} body weight was orally administrated to adult rats. They were killed either 2 or 10 days post-treatment. The primary defects were the fragmentation of the head caps, abnormal vesicles that failed to fuse to developing acrosomes, lack of acrosomic granules and disruption of golgi apparatus. In later stages, cytoplasmic vacuolization and mitochondrial and nuclear malformation were apparent. These changes were particularly prominent with the higher dose and the longer post-treatment interval. The results indicate that the treatment of rats with the exogenous melatonin produces a variety of acrosomal abnormalities as well as other cellular changes during early spermiogenesis. The possible mechanisms leading to these melatonin-induced morphological defects in early spermatids are discussed.

Key words: Melatonin, early spermatids, ultrastructure, acrosome, golgi apparatus, Egypt

INTRODUCTION

Melatonin is an indole derivative neuroendocrine hormone that is secreted principally by pineal gland at night. It works in a variety of ways as a circadian rhythm modulator, endocrine modulator and immunomodulator. There are increasing concerns about the use of exogenous melatonin as a potential treatment for circadian rhythm, sleep disorders and depression (Koster-Van Hoffen *et al*., 1993; Rao *et al*., 1994; Miyamoto *et al*., 1999; Dagan and Ayalon, 2005; Wassmer and Whitehouse, 2006; Skene and Arendt, 2007). Growing use of melatonin as a protective agent against certain harmful effects resulted from treatment of animals and humans with many compounds such as chlorpyrifos-ethyl and lipopolysaccharides was demonstrated (Gultekin *et al*., 2001; Nava and Carta, 2001; Elmegeed *et al*., 2008). However, melatonin was reported to be toxic to mammalian male reproductive organs. Testicular damage by this hormone involved lack of full spermatogenesis (Rissman, 1980; Ng and Ooi, 1990), testicular atrophy (Tamarkin *et al*., 1976; Rudeen, 1981; Lang *et al*., 1984; Hiebert *et al*., 2006) and infertility (Tamarkin *et al*., 1976; Yamada, 1992). In addition to these adverse effects, Rashed *et al*. (2010) reported that the administration of melatonin to rats resulted in various morphological abnormalities in spermatogenic cells including paucity and degeneration of late spermatids and exfoliation of early spermatids and primary spermatocytes into the seminiferous tubular lumina.

In the course of the first seven steps of mammalian spermiogenesis, the Golgi apparatus contributes actively to the formation of the acrosomic system which caps the surface of the nucleus. After completing the formation of the acrosomic system of spermatids, Golgi apparatus separates from it and migrates towards the caudal pole of the cells (Susi *et al*., 1971; Clermont and Tang, 1985). Following the first seven steps of spermiogenesis, during the acrosome phase, the nuclei of spermatids begin to elongate. The acrosome reaches its final shape at the end of the maturation phase during the formation of late spermatids. Most of the cytoplasm and organelles of late spermatids are eliminated in the residual body which is phagocytized and digested by the Sertoli cell (Russell *et al*., 1990). In spermatids of Golgi and cap phases of spermiogenesis, Golgi-derived materials are mainly transported to the developing acrosome via Golgi derived vesicles and this pathway is called Golgi to acrosome vesicular trafficking (Moreno *et al*., 2000). The present study was conducted in order to examine the effects of exogenous melatonin on rat spermatids during early spermiogenesis with a special emphasis on acrosome development using electron microscopy.

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MATERIALS AND METHODS

Animals: About 36 adult male Sprague-Dawley rats weighing 170-200 g were used in this study. All rats were healthy and kept in clean and good aerated animal cages at 25±2°C, relative humidity of 45-50% and 12 h light-cycle with food and water available *ad libitum*. This investigation was approved and conducted in accordance with the ethical guidelines set forth by the University of Ain Shams Committee for Care and Use of Laboratory Animals.

Chemicals: Melatonin was purchased from Sigma (St Louis, MO, USA) and dissolved in 1.0 mL ethanol and diluted with 99 mL physiological saline (1%, v/v) as vehicle at final concentration 0.01 mg mL^{-1}. All other chemicals were from either Sigma or BDH.

Experimental design: The rats were divided randomly into four groups (groups A-D). Groups A and B (6 rats each) served as control and vehicle-carrier, respectively. The other two groups (12 rats each) were used for treatment with melatonin. One dose of melatonin was administrated orally through a stomach tube to the rats of groups C and D at doses of 0.05 and 0.1 mg kg^{-1} b/wt, respectively. Since, the animals secrete the endogenous melatonin during the night, the exogenous melatonin was given to the rats at the onset of night. Six rats from each group (C and D) were sacrificed after 2 days. The other six rats of these two groups were sacrificed 10 days post melatonin administration. The testes of all groups were dissected out for ultrastructural examinations.

Tissue processing for electron microscopy: Testes were excised and washed in cacodylate buffered glutaraldehyde fixative. After removing the tunica albuginea, the tubules were dissected out, divided into small pieces and immediately fixed in 3% glutaraldehyde buffered with 0.1 M cacodylate buffer for 4 h at 4°C. They were washed in 0.1 M cacodylate buffer overnight, post-fixed in cacodylate buffered 1% osmium tetroxide for 2 h, dehydrated in graded series of ethyl alcohol and were then cleared in propylene oxide. The tissues were embedded in epon (Glauret and Lewis, 1998). Ultrathin sections were cut with an ultramicrotome (American Optical Corporation, USA), stained with uranyl acetate and lead citrate and were then examined with a transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Control and vehicle-treated rats: Electron microscopic examination of early spermatids from the vehicle-treated rats revealed normal morphological characteristics similar

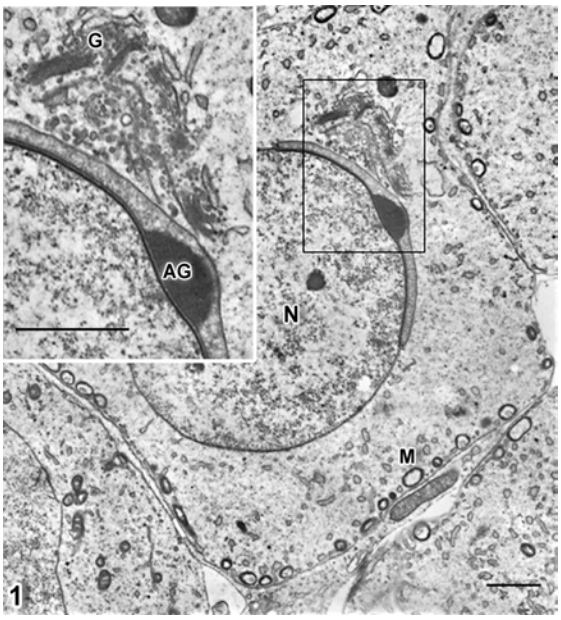

Fig. 1: An electron micrograph of a normal step 6 spermatid. This spermatid has well-developed acrosomic system with a prominent Acrosomic Granule (AG) and hemispherical Golgi apparatus (G). The cytoplasm contains a centrally located spherical Nucleus (N) and peripherally placed Mitochondria (M). Scale bar = 1 μm

to those of control ones where their nuclei are spherical and located in the center of the cells. The Endoplasmic Reticulum (ER) consists of scattered distended cisternae with few or no ribosomes at their surfaces. A layer of mitochondria beneath the plasma membrane that are associated with flattened cisternae of ER is also a characteristic feature of these germ cells. The Golgi apparatus shows one or several stacks of flattened saccules which run in parallel. Some saccules bridge adjacent stacks. These stacks seem to be roughly spherical in step 1 spermatids but take on a hemispherical shape capping the acrosomic system in steps 2-7 spermatids. The Golgi apparatus is closely associated with a network of ER cisternae at both cis and trans-faces. The latter face is also associated with a loose aggregate of coated and noncoated vesicles and vacuoles. The acrosomic system is associated with the nuclear envelope which exhibits a thickening at the area of contact (Fig. 1).

Rats received 0.05 mg kg^{-1} body weight: In rats received 0.05 mg kg^{-1} b/wt of melatonin and sacrificed after 2 days, abnormalities of acrosome development in some spermatids at steps 4 and 5 were identified by the presence of acrosomic system with no acrosomic granule

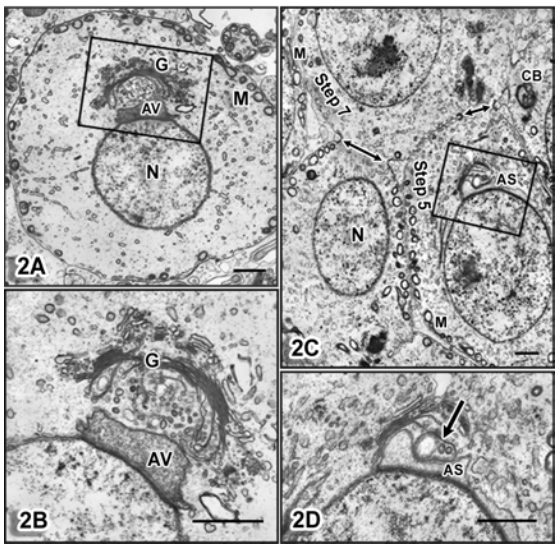

Fig. 2: Electron micrographs of spermatids from melatonin-treated rats at 2 days after administration with 0.05 mg kg^{-1} body weight. A, step 4 spermatid shows abnormal Acrosomic Vesicle (AV) with no acrosomic granule. B, enlarged part of a (the rectangle). C, asynchronized spermatid development in stage VII; steps 5 and 7 spermatids are unusually associated. Note the stretching of some Mitochondria (M) and widening of the intercellular bridges (double arrows). D, enlarged part of C (the rectangle). Step 5 spermatid shows halve acrosomic system with no acrosomic granule and a discontinuous Head Cap (HC). Some transit vesicles are seen enwrapped by Golgi saccules (arrows). AS, Acrosomic System; g, Golgi apparatus; N, Nucleus; CB, Chromatoid Body. Scale bar = 1 μm

(Fig. 2, 3a). The outer acrosomal membrane lost its regular outline. The trans-side saccules of the Golgi apparatus were seen to envelope the transit vesicles in steps 5-7 spermatids (Fig. 2d, 3b). Occasional vacuoles of various sizes were observed in the head caps in close contact with the acrosomic granules in steps 5 and 7 spermatids in stage V and VII, respectively (Fig. 3a, b). Spermatids with a discontinuous head cap were occasionally observed on treatment with this dose. Asynchronized spermatid development was frequently noticed as steps 5 and 7 spermatids were abnormally associated within the same stage (Fig. 2c). Other cellular abnormalities included elongation and stretching of mitochondria with loss of their cristae and their abnormal distribution throughout the cytoplasm, widening of the intercellular bridges and disruption of the nuclear envelope.

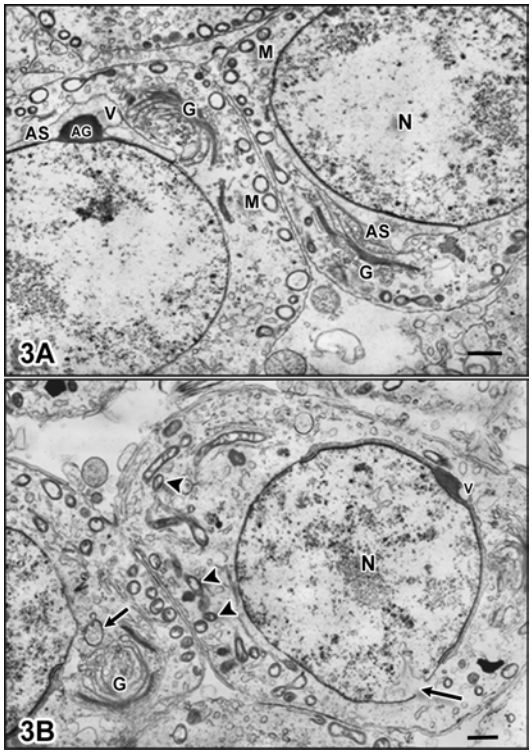

Fig. 3: Electron micrographs of spermatids from melatonin-treated rats at 2 days after low dosage. A, step 5 spermatids. Note the absence of acrosomic granule in one spermatid and the occurrence of Vacuoles (V) in a dilated acrosomic system of the other one. B, step 7 spermatid. Vacuole formation within the acrosomic system is evident. Note the disruption of the nuclear envelope (long arrow) and the abnormal distribution of mitochondria throughout the cytoplasm (arrowheads). Some transit vesicles are seen enwrapped by Golgi saccules (short arrow). G, Golgi apparatus; AS, Acrosomic System; AG, Acrosomic Granule; M, Mitochondria; N, Nucleus. Scale bar = 1 μm

The severity of degenerative effects of melatonin on the early spermatids was increased at 10 days post melatonin treatment. Spermatids with a fragmented acrosomic system were more pronounced (Fig. 4a, b). The less-developed acrosomic system was also observed to lack the acrosomic granule. As the acroplaxome, the dense filamentous elements found between the inner acrosomal membrane and the nuclear envelope was disrupted, the malformed acrosome partially lost its adhesion to the nucleus with the occurrence of small vesicles between the two organelles (Fig. 4c). At the

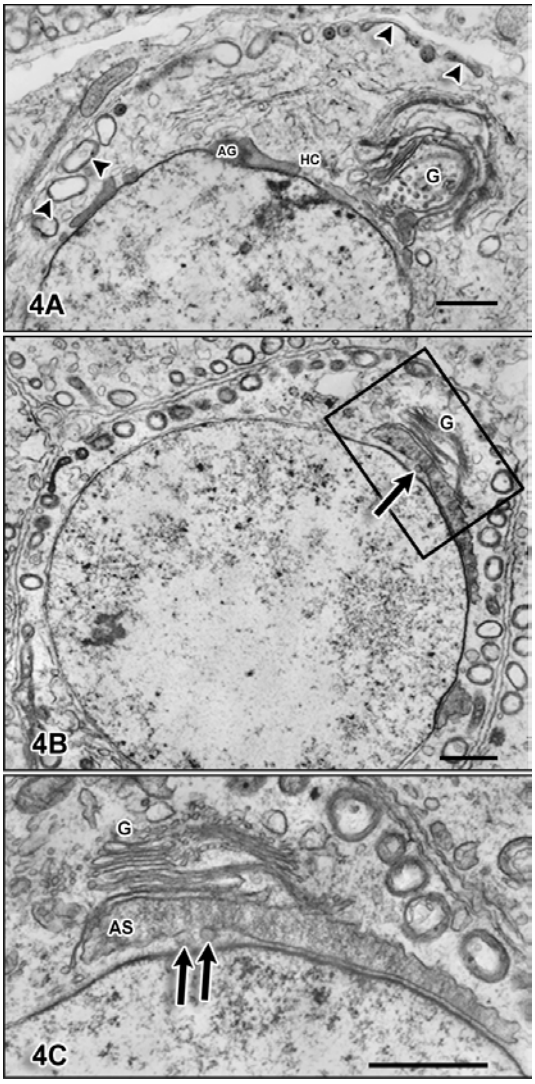

Fig. 4: Electron micrographs of spermatids from melatonin-treated rats at 10 days after low dosage. A, step 7 spermatid shows a fragmented Head Cap (HC). Stretched and dilated mitochondria (arrowheads) are evident. B, step 7 spermatid with a fragmented Acrosomic System (AS) that lacks the acrosomic granule. C, enlarged part of B (the rectangle). The abnormally formed acrosome partially looses its adhesion to the nucleus with the presence of small vesicles (arrows) in the disrupted acroplaxome between the two organelles. Note that Golgi apparatus (G) looses the ER elements and the vesicular profile at its trans-face. Scale bar = 1 μm

trans-face of the Golgi apparatus of some spermatids, the network of ER cisternae and the vesicular profile disappeared leading to direct connection between the trans-side saccules and the outer acrosomal membrane in

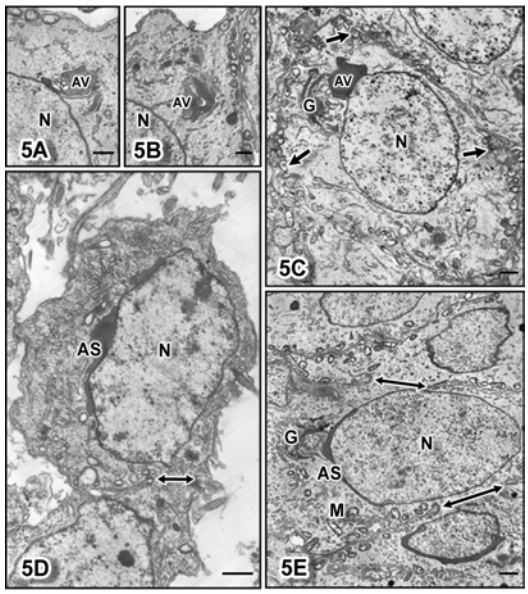

Fig. 5: Electron micrographs of spermatids from melatonin-treated rats at 2 days after treatment with 0.1 mg kg^{-1} body weight. A and B, the Acrosomic Vesicle (AV) of step 3 spermatids are deformed and separated from the underlying Nucleus (N). C, step 3 spermatid. The Golgi apparatus (G) looses its organization and becomes degenerated. The nuclei show irregular outline and marginal chromatin condensation. Aberrant mitochondria (arrows) with central rings are noticed. D and E, step 7 spermatids. The Acrosomic System (AS) is fragmented and/or contains a homogeneous dense matrix. Malformation of the nuclei and abnormal arrangement of Mitochondria (M) are pronounced. Note the widening of the intercellular bridges (double arrows). Scale bar = 1 μm

step 7 spermatids in stage VII (Fig. 4c). Nuclei of some spermatids with abnormally formed acrosomes showed chromatin disintegration and irregularity of their nuclear envelopes. Both stretched and dilated mitochondria were seen with this post-treatment interval.

Rats received 0.1 mg kg^{-1} body weight: Anti-spermatid effects of melatonin at 0.1 mg kg^{-1} b/wt were similar to those observed with the lower dose. However, more severe disruption was observed. The most notable change in early spermatids ultrastructure in melatonin-treated rats 2 days post administration was the occurrence of abnormal acrosomic system in step 7 spermatids which displayed a fragmented head cap containing a homogeneous dense matrix. This dense matrix was also observed allover in the acrosomic vesicle of step 3 spermatids (Fig. 5c, d). The acrosomic vesicle of step 3

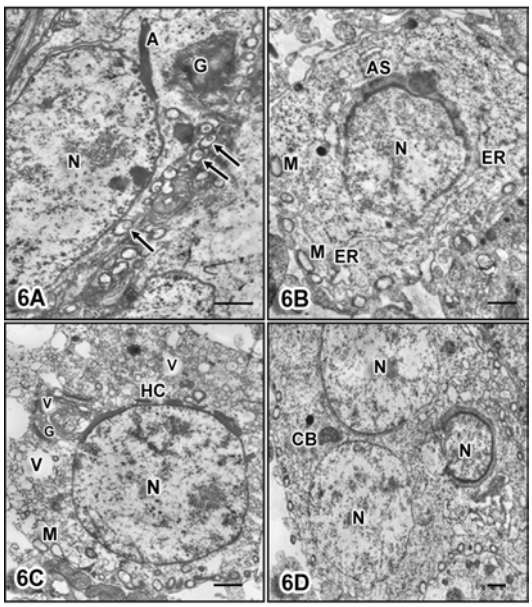

Fig. 6: Electron micrographs of spermatids from rats received a high dose of melatonin at 10 days post treatment. A, step 3 spermatid shows flat Acrosome (A) that is partially detached from the underlying malformed nucleus. Degenerated Golgi apparatus (G) and mitochondria with central rings (arrows) are apparent. B and C, step 7 spermatids display malformed and fragmented Head Cap (HC). Disruption of the nuclear envelope is severe. Dilation of ER and cytoplasmic Vacuolization (V) are evident. D, multinucleated giant cell. AS, Acrosomic System; N, Nucleus; M, Mitochondria; CB, chromatoid body. Scale bar = 1 μm

spermatids occasionally lost its adhesion to the nucleus either partially or completely. Direct connection between the Golgi saccules and the outer acrosomal membrane similar to those seen with the dose 0.05 mg kg^{-1} b/wt was observed (Fig. 5a, b). In some spermatids, the Golgi apparatus lost its organization and became degenerated (Fig. 5c). Malformation of the nuclei was become more severe; they showed irregular outline and chromatin condensation at the margin (Fig. 5c-e). Mitochondria with a central ring formation were noticed. In some spermatids, loss of mitochondria was evident. Widening of the intercellular bridges was more pronounced (Fig. 5e).

In rats treated with the higher dose of melatonin and sacrificed after 10 days, the morphological defects of early spermatids became more evident. The cytoplasm of such spermatids exhibited marked fragmentation and dilation of ER elements and significant vacuolization (Fig. 6b, c). Disruption of the nuclear envelope became more severe. Chromatin condensation subjacent to the nuclear envelope was evident. The number of altered mitochondria with a central ring formation was remarkably increased. Spermatids showed various degrees of malformation of their acrosomic system and Golgi apparatus. These included a marked fragmentation of the head cap of step 7 spermatids and hypodevelopment of the acrosome of step 4 spermatids. Flat acrosome being partially detached from the underlying nucleus was also observed in step 3 spermatids (Fig. 6a). In some spermatids, Golgi apparatus revealed again direct connection between its saccules and the outer membrane of the fragmented head cap. Vacuolization within the cis and trans-elements of the Golgi apparatus was pronounced (Fig. 6c). The Golgi apparatus of other spermatids was disorganized. This disorganization was characterized by loss of Golgi stacks and saccules and their associated vesicles, vacuoles and ER elements (Fig. 6a). Multinucleated giant cells were observed with this post-treatment interval (Fig. 6d).

DISCUSSION

Spermatogenesis of the rat testis has been classified into 14 stages based upon the possible germ cell association within the seminiferous tubules. Cohorts of spermatids in each stage show a synchronized development of acrosome (Leblond and Clermont, 1952; Hess, 1990). The biogenesis of the acrosome takes place during the initial phase of spermatid development from Golgi apparatus. In spermatids, Golgi apparatus is an array of cisternal membrane structures that are arranged in stacks. These stacks can be organized end to end to form a continuous network structure. Golgi stacks are extremely dynamic structures through which large amounts of secretory materials and membranes pass on their way to many destinations within the cell. Acrosome biogenesis during Golgi phase takes place when numerous proacrosomic vesicles, each containing a dense proacrosomic granule are formed from the concave region near the trans-Golgi saccules (termed medulla). These proacrosomic vesicles fuse with each other to form a single large acrosomic vesicle that associates with the nuclear envelope. During the subsequent cap phase as Golgi actively producing and delivering more proteins and membranes, the acrosomic vesicle increases in size and begins to spread over the anterior nuclear pole forming the so called acrosomic system (Tang *et al*., 1982).

In the present study, spermatids with various acrosome abnormalities were observed with the two melatonin doses used at both days 2 and 10 post

treatment. The primary defect of acrosome formation seemed to be the lack of acrosomic granule and fragmentation of the head cap. This suggests that the acrosomic granules were synthesized and accumulated in the Golgi apparatus but were prohibited from reaching the acrosomes. Immunocytochemical analysis on the effects of carbendazim on the acrosome of rat using antibody MN7 by Nakai *et al.* (1998) showed that the reaction products of MN7 were synthesized and accumulated in the Golgi apparatus but were not delivered to the acrosome. The study of Lin *et al.* (2007) has demonstrated that the absence of ZPBP1 (Zona Pellucida Binding Proteins in the acrosome) and its novel paralog ZPBP2 prevents proper acrosome development resulting in acrosome fragmentation and aberrant acrosomal membrane outline. Some spermatids which lacked acrosomic granules in the present study showed various impairments of acrosome formation including biogenesis of half acrosomes. The underlying mechanism of this abnormality might be explained on the basis of the failure of the acrosomic vesicles to fuse with the developing acrosome. Indeed, some acrosomic vesicles were seen surrounded by elements of Golgi saccules. In some step 7 spermatids, Golgi vesicles were often observed adjacent to the disrupted area of acroplaxome. These vesicles most likely are not the fragmented acrosomic system but rather caused by their defective fusion to the developing acrosome.

In some extreme cases, the Golgi apparatus lost its organization and became vacuolated and degenerated. The loss of Golgi apparatus or even its medullary component appears to affect significantly the overall structure of the acrosomic system. This portion of the Golgi apparatus is a station in which Golgi-derived materials appear to temporarily accumulate before being released to the acrosomic system (Clermont and Tang, 1985). In some step 3 spermatids with a degenerated Golgi apparatus, flat acrosomic vesicles were seen detached from the underlying disrupted acroplaxome. Similar changes have been reported in acrosome-deficient Hrb mice mutants (Kierszenbaum and Tres, 2004). Hrb is a protein associated with the proacrosomal transporting vesicles in round spermatids. Thus, melatonin might disrupt such proteins which were suggested to function in vesicle-to-vesicle fusion during acrosome formation.

Another impairment included spermatids with acrosomes that partially fail to expand over the nuclei in stage VII. This finding indicates that in testis treated with melatonin, some spermatids arrest their acrosome development at a level of approximately step 5 in stage VII. Similar findings have been reported by Nakai and Hess (1997) and Nakai *et al.* (1998) who demonstrated asynchronization of acrosome development during early phases of spermiogenesis in rats treated with the fungicide cabendazim.

Occasional acrosomes with an electron dense matrix were observed in spermatids at steps 3 and 7. Acrosomic system with an electron dense matrix normally begins in step 9 spermatids when the acrosome forms tight association with the Sertoli cell and anchor the nucleus to the junctional structures (Russell *et al.*, 1990). Moreno *et al.* (2006) have shown that the microtubule network in mice spermatids is involved in the position/structure and sorting of Golgi-derived vesicles to specific cell components. They have proposed that alterations in the organization of the spermatid cytoskeleton may provoke missorting of the proteins to the wrong cell components. This missorting may explain the pre-mature condensation of the acrosomal matrix that is some Golgi derived proteins whose final destination is elsewhere within the cell may reach incorrectly the acrosome. Besides, melatonin may affect the proteins which regulate condensation of the acrosomal matrix, altering concomitant condensation of the matrix with the proper spermatid steps.

Cytoplasmic vesicles are transported to their final destinations within cells via tracks of microtubules (Lodish *et al.*, 1995). Active trafficking from the Golgi apparatus is mediated by vesicular carriers and transient tubular connections (Lupashin and Sztul, 2005; Moreno *et al.*, 2006). The present study indicates that melatonin may disrupt the elements, possibly microtubules, responsible for the maintenance and functioning of the Golgi apparatus and its location in relation to other organelles to ensure a normal flow of materials into and away from its saccules. Moreno *et al.* (2000) have localized COPI (a vesicle coat protein complex) and Golgin-97 and Golgin-95/GM130 (proteins that are involved in post-Golgi trafficking) both in the Golgi apparatus and in the acrosomal membrane in Golgi and cap phases of mammalian spermatids suggesting a role for these proteins in vesicular and/or tubular trafficking between the two organelles. Many golgins function in a variety of membrane-membrane and membrane-cytoskeleton tethering events (Barr and Short, 2003). Haraguchi *et al.* (2004) have localized the ubiquitin signals in both Golgi vesicles and the acrosomic system. They have suggested a vesicular transport pathway through which the ubiquitin is involved in the selective transport of proteins between the Golgi apparatus and the

acrosome. It can be therefore, speculated in melatonin-treated testis that the degrees of acrosome abnormalities depend on how much Golgi to acrosome vesicular and/or tubular trafficking is disrupted and how much materials delivered from Golgi apparatus to acrosome are perturbed.

During spermiogenesis, the association of the acrosome with the underlying nucleus is accomplished by the acroplaxome, an actin-keratin-containing structure localized between the two organelles (Kierszenbaum *et al*., 2003). This acroplaxome is likely involved in the formation and maintenance of nuclear polarity in spermatids during chromatin condensation and nuclear shaping. Disruption of the acroplaxome results in separation of acrosome from its perinuclear position and nuclear deformations including elongation of the nucleus of early spermatids.

Admnistration of melatonin seems most likely to induce disassembly of cytoskeleton and disorganization of the mitochondria, resulting in their dispersion throughout the cytoplasm instead of being distributed as one layer under the cell membrane. This disorganization is followed by vesiculation of the endoplasmic reticulum and its dilation leading eventually to vacuolization of the cytoplasm. Similar ultrastructural changes in spermatids were observed in adult male rats after treatment with the indenopyridine CDB-4022 (Hild *et al*., 2007).

Formation of multinucleated giant cells in the present study has been repeatedly reported in various experimental studies, testosterone-treated and hypophysectomized rats (Muffly *et al*., 1994), β-estradiole-3-benzoate or bisphenol A-treated rats and mice (Toyama *et al*., 2001; Toyama and Yuasa, 2004; Toyama *et al*., 2004). Actin filaments may participate in stabilizing intercellular bridges (Russell *et al*., 1987). The disruptive effect of melatonin on this stabilizing region leads to progressive widening of the intercellular bridges resulting in the establishing of multinucleated giant cells formed mainly from fused early spermatids.

It has been demonstrated for many years that testosterone is a necessary hormone involved in the regulation and maintenance of spermatogenesis in adult testis. Melatonin has been shown to reduce LH-stimulated testosterone secretion by inhibiting adenyl cyclase activity. However, melatonin is also likely to influence non-cAMP mediated testosterone secretion as it reduces Gonadotropin-Releasing Hormone (GnRH)-dependent testosterone secretion (Valenti *et al*., 1997). A comparable effect of melatonin has been noted in the decrease of GnRH-induced LH secretion from gonadotrophs of neonatal and adult rats (Vanececk, 1998; Yilmaz *et al*., 2000). The study of Valenti *et al*. (1999) on the adult rat leydig cells cultured *in vitro* has shown that melatonin inhibits GnRH-dependent testosterone secretion by:

- Decreasing Ca^{2+} concentration through impairment of the GnRH-dependent release of Ca^{2+} from intracellular stores
- Blocking the enzyme activity along the steroidogenic pathway

Thus, melatonin-induced disruption of the microtubule-dependent Golgi to acrosome pathway and other morphological defects in early spermatids are likely effects that occur due to:

- Impairment of testosterone secretion
- Direct consequence of changes in Ca^{2+} concentration in early spermatids

CONCLUSION

In the present study suggests that a single, low or high dose of orally administrated melatonin at different post-treatment intervals results in a perturbation of the Golgi to acrosome trafficking in early spermatids. Disruption of this pathway causes prohibition of materials delivered from the Golgi apparatus to the acrosome and impairment of acrosome development. This disruption also reaches other cellular components leading to their deformation and disorganization. Additional immunocytochemical and physiological studies remain to be investigated to provide important clues to understanding in depth the mechanisms underlying the morphological changes in spermatids of melatonin-treated rats.

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