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Relationship between the nucleolar cycle and chromatoid body formation in the spermatogenesis of *Phrynops geoffroanus* (Reptilia Testudines)

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The nucleolus is a distinct nuclear territory involved in the compartmentalization of nuclear functions. There is some evidence of a relationship between nuclear fragmentation during spermatogenesis and chromatoid body (CB) formation. The CB is a typical cytoplasmic organelle of haploid germ cells, and is involved in RNA and protein accumulation for later germ-cell differentiation. The goal of this study was to qualitatively and quantitatively describe the nucleolar cycle during the spermatogenesis of *Phrynops geoffroanus* (Reptilia Testudines), and compare this nucleolar fragmentation with CB formation in this species through the use of cytochemical and ultrastructural analysis. Qualitative analysis showed a fragmentation of the nuclear material after pachytene of the first meiotic division in the primary spermatocytes. Quantitative analysis of the nucleolar cycle revealed a significant difference in the number of nucleoli and in the size of the nucleolus between spermatogonia and early spermatids. Using ultrastructural analysis, we recorded the beginning of the CB formation process in the cytoplasm of primary spermatocytes at the same time as when nuclear fragmentation occurs. In the cytoplasm of primary spermatocytes, the CB was observed in association with mitochondrial aggregates and the Golgi complex. In the cytoplasm of early spermatids, the CB was observed in association with lipid droplets. In conclusion, our data show that the nucleolus plays a role in the CB formation process. During spermatogenesis of *P. geoffroanus*, the CB is involved in some important biological processes, including acrosome formation and mitochondrial migration to the spermatozoon tail and middle piece region.

Keywords: nucleolar cycle; spermatogenesis; chromatoid body; reptiles

1. Introduction

The nucleolus is a distinct nuclear territory involved in the compartmentalization of nuclear functions (Hernandez-Verdun 1991). Most events of ribosome biogenesis, such as ribosomal RNA synthesis, processing, and ribosome subunit assembly, take place within the nucleoli (Cmarko et al. 2008). The machineries of ribosome biogenesis are distributed into the three main nucleolar compartments: the fibrillar centers (FCs), the dense fibrillar components (DFCs), and the granular components (GCs). The location of the nucleolar machineries depends on their function in the production of both small and large ribosome subunits. Ribosomal RNAs are synthesized, processed, and assembled with ribosomal proteins in these three nucleolar compartments (Sirri et al. 2008). Therefore, ribosome biogenesis is the main function attributed to the nucleolus (Gerbi et al. 2003; Boisvert et al. 2007; Sirri et al. 2008). However, many studies have shown that the nucleolus also has non-ribosomal functions, including nucleotide modifications of several small RNAs, biosynthesis of the signal recognition particle and phased sequestration, and the release of proteins involved in gene silencing, senescence and cell division (Pederson 2002). Moreover, some proteins that are involved in the control of the cell cycle, such as Net1, Cdc14 and Sir2, are located in the nucleolar compartments (Shou et al. 1999; Straight et al. 1999; Visitin et al. 1999; Garcia and Pillus 1999), along with the tumor suppressor proteins ARF (Carmo-Fonseca et al. 2000), p53 and Myc (Montanaro et al. 2007). The nucleolus of germ cells seems to be involved in meiotic cell cycle control, because most of the Pch2 protein, which is required for the meiotic checkpoint that prevents chromosome segregation when recombination and chromosome synapses are defective, localizes to the nucleolus (San-Segundo and Roeder 1999).

Many studies in the literature describe the relationship between nucleolar material fragmentation during the spermatogenesis process and chromatoid body (CB) formation (Comings and Okada 1972; Andersen 1978; Andonov 1990; Peruquetti et al. 2008, Peruquetti et al. 2010a, Peruquetti et al. 2011). The CB is a typical cytoplasmic organelle of haploid germ cells, and is involved in RNA and protein accumulation for later germ-cell differentiation (Söderström and Parvinen 1976; Saunders et al. 1992). Some recent studies indicate that the CB is a highly specialized structure that may function as an intracellular focal domain which organizes and controls RNA processing in male germ cells. These findings suggest that the CB acts as a subcellular coordinator of different RNA-processing pathways, and centralizes post-transcriptional mRNA control in the cytoplasm of haploid male germ cells (Parvinen 2005; Kotaja et al. 2006; Kotaja and

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Sassone-Corsi 2007). However, some authors found that the CBs have some aggresomal features, which suggest that they are not a synthetic site as proposed previously, but are instead a degradation site where unnecessary DNA, RNA, and proteins are digested (Haraguchi et al. 2005). Despite many efforts to elucidate it, it is not clear what the main function of this cytoplasmic structure of germ cells is. Its origin is still uncertain, though many theories exist. Some authors suggest that the origin of the CB is nuclear (Parvinen and Parvinen 1979; Parvinen et al. 1997). Others have proposed that the CB precursor is a dense interstitial material occurring between mitochondrial clusters (Fawcett et al. 1970), or that it is a result of the mitochondrial products that are released in the cell cytoplasm (Reunov et al. 2000). However, still others have suggested that the CB originates from nuages, a ribonucleoproteic complex derived from the nucleolus, which migrates to the cytoplasm during earlier spermatogenesis (Comings and Okada 1972; Andersen 1978; Andonov 1990; Peruquetti et al. 2008, Peruquetti et al. 2010a).

In light of these questions, the goal of this study was to qualitatively and quantitatively characterize the nucleolar cycle during the spermatogenesis of *Phrynops geoffroanus* (Reptilia Testudines) and to compare the nucleolar fragmentation with CB formation in this species using cytochemical and ultrastructural analysis. Among the Testudines reptiles, the species chosen represents the most primitive amniotic vertebrates. In addition to the amnion, some reproductive modifications, particularly in the male, have accompanied the species during the transition from water to land and through the processes of their independence from water for breeding (Gribbins et al. 2003). Because they belong to a transitional from-water-to-land group, reptiles show some reproductive characteristics that are similar to amniotic vertebrates (birds and mammals), such as tubular testis and internal fertilization (with its associated copulatory organs and an organ for sperm storage, or the epididymis) (Pudney 1990). Reptiles also share reproductive characteristics with amniotes vertebrates (fish and amphibians), such as external fertilization and cystic testes, where sperm develop in pouches containing Sertoli cells (Gribbins et al. 2003). Most of the studies related to CB characterization were completed using invertebrates, fish, amphibians, and mammals as biological models. Thus, another purpose of this study was to add information to the literature about CB formation and its probable functions during the spermatogenesis of reptiles, an animal group that possesses interesting reproductive characteristics.

2. Material and methods

Testes from five adult males of *Phrynops geoffroanus* (Reptilia Testudines), which were collected between April 2005 and May 2006 at the Felicidade streamlet (in the city of Sao Jose do Rio Preto, in the state of Sao Paulo, Brazil), were used in this study.

All of the animals were transported to the Anatomy Laboratory (Department of Biology, Sao Paulo State University – UNESP/IBILCE) and acclimatized in appropriated terrariums. The animals received food and water ad libitum during the acclimatizing period. The methods for the anesthesia and euthanasia are described in Cabral et al. (2011). After their deaths, the animals were dissected and their gonads were removed and prepared for each biological analysis.

Cytochemical analysis

The testes of each animal were removed and immersed in a Bouin fixative solution for 24 hours. The material was embedded in glycol-metacrylate historesin (Historesin Leica®). Sections (1–3 μm thick) were obtained in a Leica RM 2155 microtome. Tissue sections were submitted to various cytological and cytochemical procedures, including: hematoxylin-eosin (HE) (*sensu* Ribeiro and Lima (2000)), toluidine blue (TB), modified Critical Electrolyte Concentration for detecting RNA (CEC) (Mello 1997), silver-ion impregnation (AgNOR) (Howell and Black 1980), and Feulgen reaction (Mello and Vidal 1980). The sections of germ epithelium were evaluated under an Olympus BX 60 photomicroscope and documented using Image Pro-Plus Media Cybernetics computer software, version 6.1 for Windows for image analysis.

In addition to the qualitative analysis of the nucleolar material distribution, the tissue sections that were subjected to silver ion impregnation were used for quantitative analysis, in order to determine the number of nucleoli in the spermatogonia and earlier spermatids, and also to measure the nuclear and nucleolar areas of these types of cells.

Determination of the number of nucleoli in the spermatogonia and earlier spermatids

The number of nucleoli was determined in all spermatogonia and earlier spermatids recorded in this study. Spermatogonia (122.6 ± 7.6) and earlier spermatids (138.0 ± 3.46) were considered from each specimen studied ($n = 5$). Due to the different amount of cells analyzed in each cell type, the percentage of the number of nucleolus in each cell type was calculated.

Measuring the nuclear and nucleolar areas of the spermatogonia and earlier spermatids

Spermatogonia and early spermatids were photodocumented using an Olympus BX 40 photomicroscope

and Image Pro-Plus Media Cybernetics computer software, version 4.5 for Windows. Next, both nuclear and nucleolar areas of these cells were measured using Image J – Image Processing and Analysis in Java software, Version 1.40 (http://rsb.info.nih.gov/ij/) for image analysis. The cells that had one single nucleolus were measured immediately, while the total area of those with two or more nucleoli was calculated by adding up the areas of each individual nucleolus.

Data analysis

Normal distribution of the dataset was tested using skewness and kurtosis analysis (Ha and Ha 2007), and variance homogenity was tested using the F max test (Zar 1999). The number of nucleoli was compared between spermatogonia and earlier spermatids, and also within the same cell type using the Two-Factor Analysis of Variance, which was complemented using the LSD multiple comparisons test (Zar 1999). Nuclear and nucleolar areas of spermatogonia were compared to nuclear and nucleolar areas of earlier spermatids using an independent *t* test (Zar 1999). Statistical significance was considered when $P \leq 0.05$.

Ultrastructural analysis

Testis fragments of each animal were removed and sliced into small pieces, and samples of the germ epithelium were cut and immersed in a 3% glutaraldehyde plus 0.25% tannic acid solution in Millonig's buffer (pH 7.3) containing 0.54% glucose for 24 hours at room temperature (Cotta-Pereira et al. 1976). After being washed with the same buffer, samples were post-fixed in 1% osmium tetroxide for 1 hour at 4°C, washed in Millonig's buffer, dehydrated in a graded acetone series, and embedded in Araldite resin. Ultrathin silver sections (50–75 nm) were cut using a diamond knife and stained with 2% alcoholic uranyl acetate for 30 min (Watson 1958), followed by 2% lead citrate in sodium hydroxide for 10 min (Venable and Coggeshall 1965). Samples were evaluated using a Leo-Zeiss 906 (Cambridge, UK) transmission electron microscope and documented using ITEM (Soft Image System – Veleta 2K×2K TEM CCD Camera) software for image analysis.

Ethics note

The animals were collect under IBAMA license number 061/2005-RAN/IBAMA. This study was approved by the Ethical Committee for Animal Research (CEEA) of Sao Paulo State University (UNESP) in Botucatu, Sao Paulo, Brazil, under protocol n°057/06.

3. Results

Cytochemical analysis

HE revealed a spermatogenesis pattern that was similar to spermatogenesis pattern of mammals, and the seminiferous tubules possessed cells at different stages of development: spermatogonia, primary spermatocytes, earlier spermatids and spermatozoon (Figure 1A). The classification of the germ cells present in the germ epithelium was made following Gribbins et al. (2003). The toluidine blue (TB) reaction revealed the cells of the male germ epithelium with an intense metachromasy in all of the nuclear domains (euchromatin, heterochromatin, nucleolus and chromosomes) (Figure 1C, 1D and 1E). The degree of metachromasy varied according to the compaction of genetic material, the ploidy of the cell nucleus, and the complexity of the nucleic acids with ribonucleoproteic (RNP) corpuscles. The TB reaction was performed as a control for the critical electrocyte concentration (CEC) variant method for RNA detection. CEC revealed spermatogonia with metachromatic nucleoli (Figure 1F), primary spermatocytes with fragmented nucleoli (Figure 1G), and earlier spermatids with no organized nucleoli (Figure 1H). Several residual bodies were detected in the seminiferous epithelium lumen. Feulgen reaction is a DNA-specific method in which all germ cell nuclei are dyed purple, and are categorized according to their degree of ploidy, functional stage, and compaction of chromatin. Heterochromatic regions were more intensively dyed and nucleoli were seen as light spots connected to these heterochromatic regions. In the spermatogonia (Figure 1I), the nucleolar area was larger than in both primary spermatocytes (Figure 1J) and earlier spermatids (Figure 1K), indicating the occurrence of fragmentation and reduction of the nucleolar area in the latter's cell type. Impregnation using silver ion (AgNOR) revealed the nucleolar regions in different germ cells. Spermatogonia were found to possess organized nucleoli (Figure 1L), primary spermatocytes possessed fragmented nucleoli (Figure 1M), and earlier spermatids possessed reorganized nucleoli (Figure 1N), although they were found to be smaller than the spermatogonium nucleoli.

Determination of the number of nucleolus in the spermatogonia and earlier spermatids

There was an interaction between the cell type and the number of nucleoli ($F = 17.247$; $P < 0.05$). The number of spermatogonia with one nucleolus was higher than the number of earlier spermatids with the same number of nucleolus. Nevertheless, the number of spermatogonia with two nucleoli was smaller than the number of

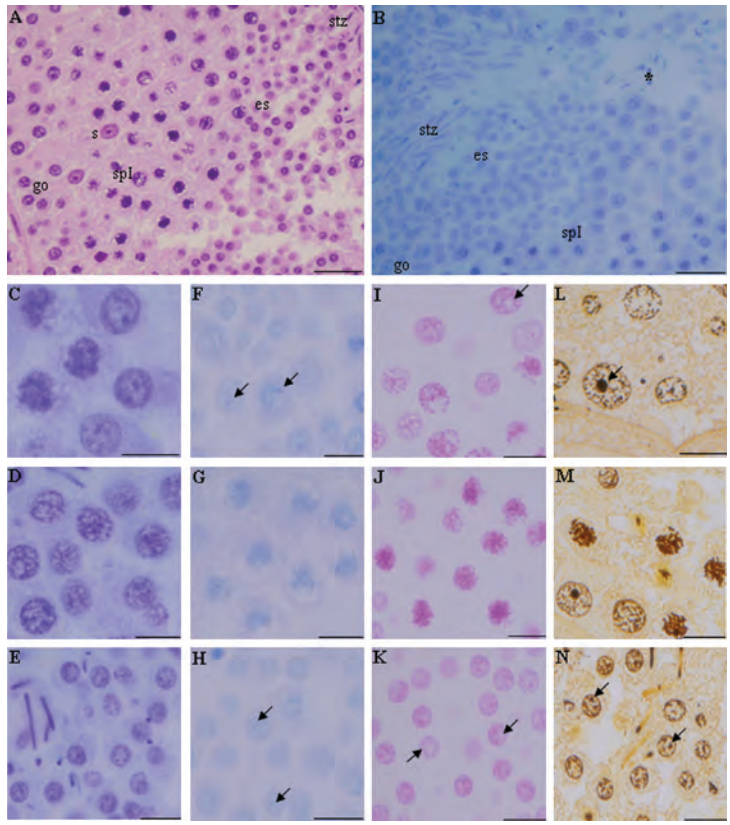

Figure 1. Cytological and cytochemical analysis of the germ epithelium of *Phrynops geoffroanus*. A: hematoxylin-eosin (HE); C–E: Toluidine blue (TB); B, F–H: modified Critical Electrolyte Concentration method (CEC); I–K: Feulgen reaction; L–N: silver ion impregnation. A and B: s, Sertoli cell; go, spermatogonia; spI, primary spermatocyte; es, earlier spermatids; stz, later spermatids; *, residual bodies. F, H, I, K, L and N: arrows, nucleolar corpuscles. Bars: A and B: 50 μm; C–N: 20 μm.

earlier spermatids with the same quantity of nucleoli (Figure 2A).

Sizes of the nuclear and nucleolar areas of the spermatogonia and earlier spermatids

There were significant differences between the nuclear areas in the spermatogonia and in the earlier spermatids ($t = 12.14$; $P < 0.05$), and also between the nucleolar area of the spermatogonia and earlier spermatids ($t = 14.36$; $P < 0.05$). In both results, the areas of the spermatogonia were larger than the areas of earlier spermatids (Figure 2B).

Ultrastructural analysis

Spermatogonia possessed compact nucleoli organized in the nucleus center, but neither ribonucleoprotein nuages nor initial chromatoid body (CB) formations were observed (Figure 3A and 3B). Some evidence suggested later nucleolar fragmentation, because organized nucleoli were still observed in the cytoplasm of primary spermatocytes in the pachytene stage, which possessed synaptonemal complex (Figure 3C). At this stage, CB formation was not observed. Complete nucleolus disorganization and the accumulation of ribonucleoproteic material were observed in the cytoplasm of primary spermatocytes in the most advanced stages. The agglomeration of this ribonucleoprotein material was found in association with mitochondrial aggregations (Figure 3D). In some cases, the material was isolated (Figure 3E), and it was less frequently associated with the Golgi complex (Figure 3F), results which suggest that the material is involved in acrosome formation. The CB of earlier spermatids was observed in association with mitochondrial aggregations, as well as with lipid droplets in the anterior nucleus region, where acrosome formation occurs (Figure 4A, 4B and 4C). Later spermatids were shown to possess a reorganized nucleolus with a reduced area. The migration of the complex composed of the CB, mitochondrial aggregations, and lipid droplets to the posterior nucleus region, where the mitochondrial sheath and spermatozoon tail formation occurs, was also observed (Figure 4D, 4E, 4F and 4G). This macromolecular complex seems to be eliminated in

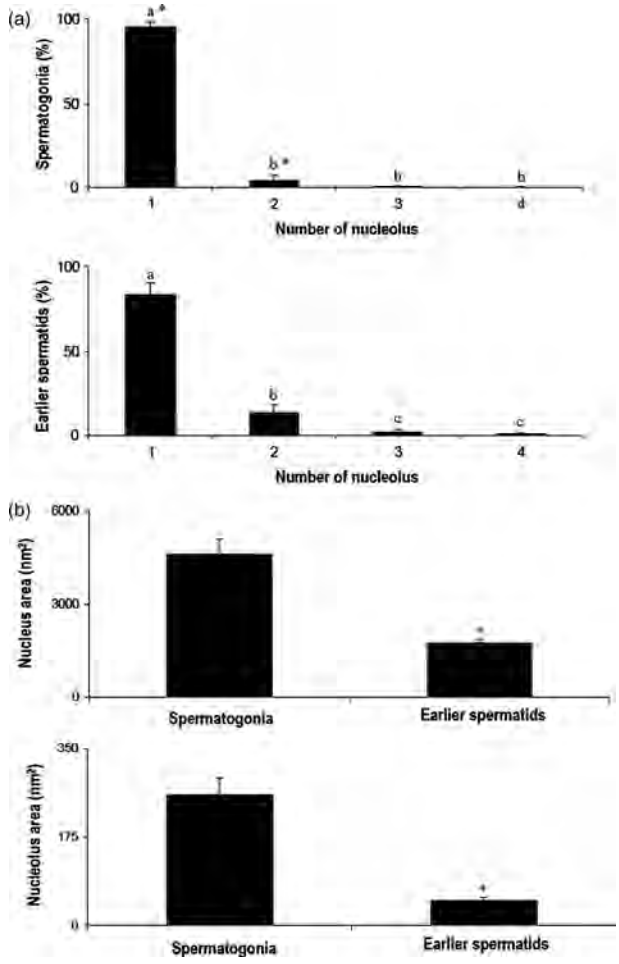

Figure 2. A: percentage of germ cells (spermatogonia and earlier spermatids) of *Phrynops geoffroanus* that possessed one to four nucleoli. The letters represent a statistically significant difference in the number of nucleoli in each cell type and the asterisk represents significant difference between both cell types (LSD, $P < 0.05$). B: areas (nm^2) of the nuclei and nucleoli of spermatogonia and earlier spermatids of *Phrynops geoffroanus.* The asterisk (*) represents a statistically significant difference between the cell types (independent t test, $P < 0.05$).

the in the seminiferous tubule lumen, participating in the formation of residual bodies (Figure 4H).

4. Discussion

The nucleolus has several crucial functions in the nucleus. It exhibits ribosome synthesis, and it is also a multifunctional nuclear domain whose activity is also linked to several pathologies (Sirri et al. 2008). In addition to the 271 nucleolar proteins involved in rRNA synthesis and processing, there are nucleolar proteins with different functions, such as the nucleotide modifications of RNAs, biosynthesis of the signal recognition particle, and the phase of sequestration and release of proteins involved in gene silencing, senescence, and cell division (Pederson 2002).

Nucleolus size increases and decreases in growing and resting cells, respectively, and it forms and disperses in every mitotic and meiotic cell-division cycle (Pikaard 2002; Teruel et al. 2007). Furthermore, most of the Pch2 protein, which is required for the meiotic checkpoint to prevent chromosome segregation when recombination and chromosome synapses are defective, localizes to the nucleolus (Garcia and Pillus 1999; San-Segundo and Roeder 1999). The qualitative analyses of the nucleolar fragmentation and distribution during *P. geoffroanus* spermatogenesis revealed that spermagonias possess one to four organized nucleoli. At the beginning of meiotic division, the primary spermatocytes in the pachytene stage possessed no visible organized nucleolus. During late meiotic division, earlier spermatids were shown to possess one to four reorganized nucleolar corpuscles, but they were smaller than the nucleoli of spermatogonia. The phenomenon of nucleolar fragmentation during prophase I and its subsequent reorganization after meiosis completion have been described in detail over

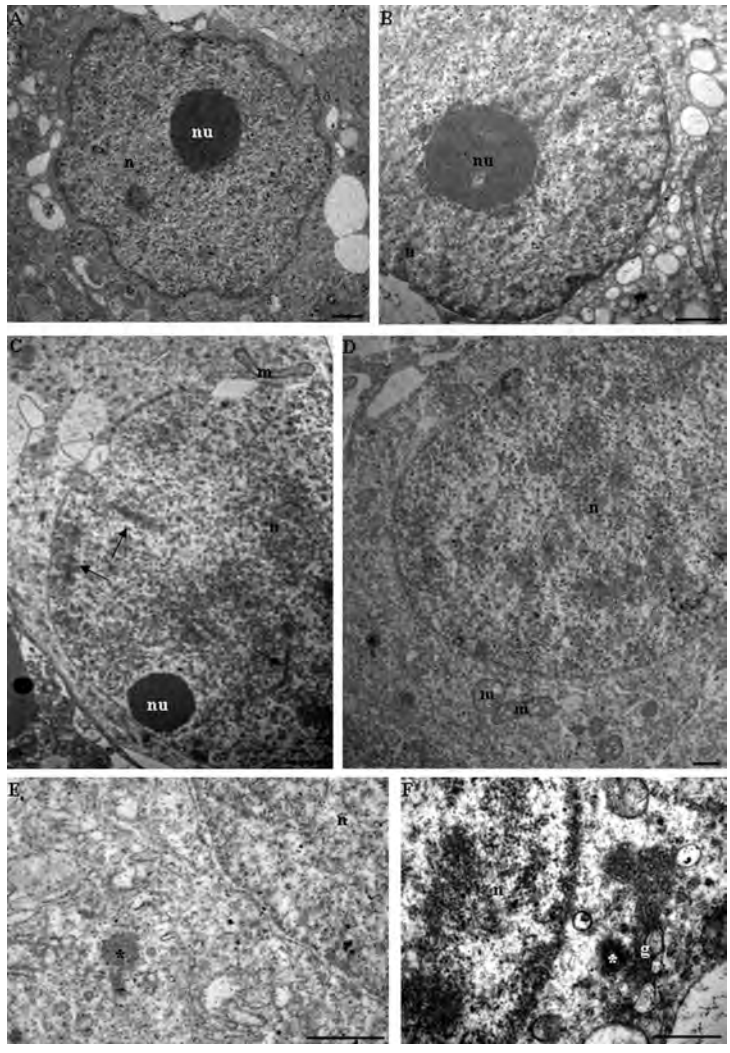

Figure 3. Ultrastructural analysis of *Phrynops geoffroanus* germ epithelium. A and B: spermatogonia with an organized compact nucleolus (nu). B: primary spermatocyte in pachytene stage with synaptonemal complex (arrows) and an organized compact nucleolus (nu). C: primary spermatocyte in pachytene stage showing nucleolus (nu) still organized. D, E and F: primary spermatocyte after the pachytene stage with no organized nucleolus and the beginning of ribonucleoprotein material accumulation in the cytoplasm (*). The ribonucleoprotein material is observed relative to mitochondrial clusters (m) and to Golgi complex (g) during the progression of the cell division. n, nucleus; nu, nucleolus; m, mitochondria; arrows, synaptonemal complex; *, chromatoid body; g, Golgi complex. Bars = 10 μm.

the past two decades (e.g. Takeuchi and Takeuchi 1990; Tartarotti and Azeredo-Oliveira 1999; Severi-Aguiar et al. 2002; Peruquetti et al. 2008, Peruquetti et al. 2010a). A decrease in the amount of C23 protein, which is responsible for rRNA transcription, and a decrease in the amount of B23 protein, which is responsible for rRNA splicing, were also observed in the nucleoli of both spermatocytes I and round spermatids (Biggiogera et al. 1991). All of these findings in different animal groups suggest that nucleolar fragmentation occurs during the beginning of meiotic prophase I, which is later reorganized in the nuclei of earlier spermatids. It has been reported that, in most chordates, there is increased nucleolar activity during prophase I, and that nucleolar activity reaches its peak during pachytene (Schmid et al. 1982; Wachtler and Stahl 1993; Teruel et al. 2007). Organized nucleoli were not observed in the nucleus of later spermatids or in mature spermatozoon. The presence of a nucleolus is not necessary for the spermatozoon, because after fecundation the nucleoli of the male and female pronuclei in the zygote are both of maternal origin. Recent studies have suggested that the maternal nucleolus, associated with other nucleoplasmic products, is essential for embryonic development (Lefrève 2008).

The number of nucleoli differed between the different germ cells analyzed, because the spermatogonia studied had one single nucleolus more frequently than the earlier

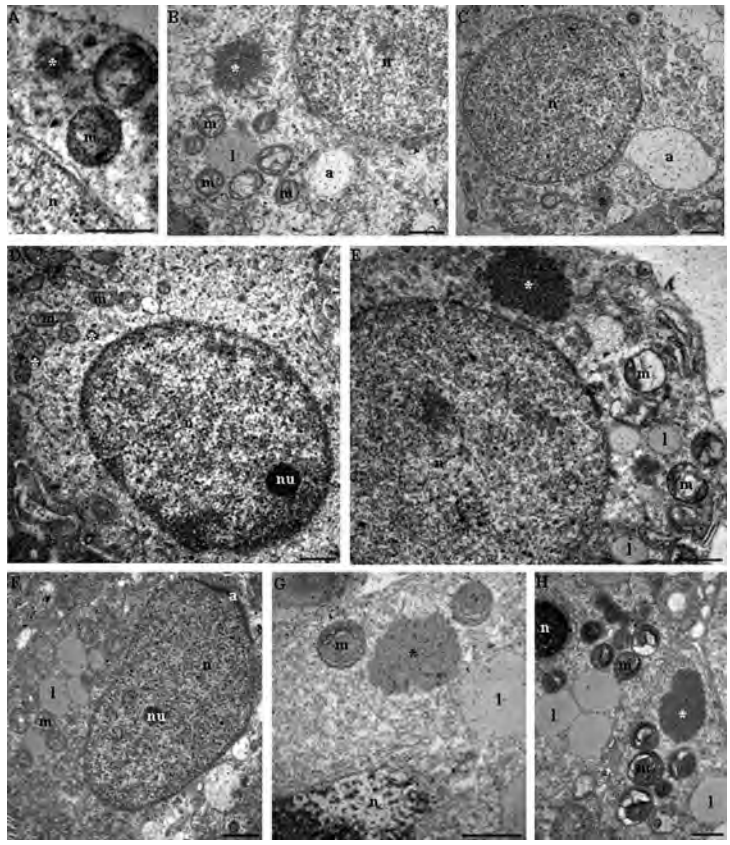

Figure 4. Ultrastructural analysis of *Phrynops geoffroanus* germ epithelium. A: earlier spermatids with CB (*) relative to mitochondria (m). B and C: earlier spermatids showing CB (*) relative to lipid droplets (l) and with mitochondrial clusters (m) near the acrosome formation (a). D, E, F , G and H later spermatids with a material that contains CB (*), mitochondrial aggregates (m) and lipid droplets (l) moving to the posterior nuclear region (n), where the mitochondrial sheath and the spermatozoon tail will be formed. CB material (*), mitochondrial clusters (m) and lipid droplets eliminated in the residual bodies. n, nucleus; nu, nucleolus; *, chromatoid body; m, mitochondria; l, lipid droplets; a, acrosomal vesicle. Bars = 10 μm.

spermatids did. Conversely, the earlier spermatids more frequently possessed two nucleoli. These results suggest an increase in the number of nucleoli during the meiotic division process. Previous studies reported that the number of nucleoli of germ cells is related to their number of RONs (Guo et al. 1996; Teruel et al. 2007). Diploid spermatogonia are therefore expected to have twice as many nucleoli as the earlier haploid spermatids. However, in this study, the opposite result was found: an increase in the number of nucleoli after meiotic division. This contradictory result can be explained, because intense nucleolar fragmentation occurs during meiotic division, which produces several nucleolar corpuscles that are highly similar to the organized nucleoli in the nuclei of spermatids. There was also a notable difference in the nucleolar area of the different germ cells. We observed that the nuclear and nucleolar areas of spermatogonia were larger when compared to the nuclear and nucleolar areas of the earlier spermatids. The size of a nucleolus is proportional to the amount of rRNA synthesized (Caspersson 1950); NOR size (the number of rRNA cistrons) is generally correlated with its expression level (Shubert and Künzel 1990); hypertrophy of the nucleolus is a state in which rRNA and ribosome synthesis has increased (Nakamoto et al. 2001); and the size of the largest nucleoli may correlate with cell division activity and also with cellular stages having high protein demand (Mosgoeller 2004). All of these findings may be related to the decrease in nucleolus size in the earlier spermatids, but its decrease can also be explained by the migration of the nucleolar fragments to the cytoplasm of germ cells in prophase I, where they may participate in CB formation.

Recent studies indicate that the CB is a highly specialized structure that may function as an intracellular focal domain which organizes and controls RNA processing in male germ cells (Kotaja et al. 2006; Kotaja and Sassone-Corsi 2007). In their studies these authors suggested a model in which the CB acts as a subcellular coordinator of different RNA-processing pathways that centralizes post-transcriptional mRNA control in the cytoplasm of haploid male germ cells.

Ultrastructural analysis revealed that the CB has a sponge-like structure, with regions that have different electron density levels (Figueroa and Burzio 1998). However, the origin of this structure remains unclear. This study was performed intending to strengthen the theory about the formation of the CB by the accumulation of proteins and other molecules coming from the fragmentation of the nucleolus.

In this study, transmission electron microscopy (TEM) indicated that spermatogonia present organized nucleoli, but that they lack ribonucleoprotein material accumulations (the precursor of CB) in the cytoplasm. Testudines represent one of the most primitive groups of amniotic vertebrates (Gribins et al. 2003), and they possess some transitive reproductive characteristics similar to amniotic vertebrates (birds and mammals) and anamniotic vertebrates (fish and amphibians). In the spermatogenesis of testudines, the CB formation process is similar to the process during the spermatogenesis of other amniotes which starts in the cytoplasm of spermatocytes I (Peruquetti et al. 2008, Peruquetti et al. 2010a). However, the CB formation process of testudines differs from that which occurs during the spermatogensis of anamniotes, which occurs in the cytoplasm of spermatogonia (Peruquetti et al. 2010b, Peruquetti et al. 2011).

The CB formation process occurs in the cytoplasm of primary spermatocytes after the pachytene stage, at the exact moment that nucleolar disorganization begins. Other studies have also reported that the CB formation process occurs during the primary spermatocyte stage (Kotaja and Sassone-Corsi 2007; Peruquetti et al. 2008, Peruquetti et al. 2010a). The CB formation process occurs through the accumulation of ribonucleoprotein material in the cytoplasm of primary spermatocytes. This ribonucleoprotein material comes together to form a single structure called the CB (Paniagua et al. 1986; Peruquetti et al. 2008, Peruquetti et al. 2010a). This ribonucleoprotein material, which plays an important role during CB formation, was observed in the cytoplasm of primary spermatocytes and was found to be associated with either mitochondrial clusters or the Golgi complex, or isolated. This association has been described in other studies, which gave rise to the hypothesis that the CB may originate from intermitochondrial material (Fawcett et al. 1970). Other authors have suggested that the CB originates from nuclear genome products, and is then supplemented by mitochondrial genome products (Reunov et al. 2000). The connection between the CB and mitochondria may also be explained by the participation of the CB in the synthesis and transport of the apocytochrome *c*, a type of cytochrome *c* isoform, which is expressed in the testis tissue (Hess et al. 1993). Therefore, in this study, we suggest that the relationship between the CB and mitochondria can be related to the migration of these structures to the caudal nuclear region, where the mitochondrial sheath and spermatozoa tail are formed. This has also been suggested by other authors (Soley 1994; Peruquetti et al. 2008, Peruquetti et al. 2010a). The association between the CB and the Golgi complex had been previously described by other authors. Söderström and Parvinen (1976), Tang et al. (1982), and Peruquetti et al. (2008, Peruquetti et al. (2010a)) suggested that the interaction between these structures could be related to acrosome formation in the earlier spermatids, because vesicles are frequently seen moving between the Golgi complex and the CB. Anton (1983) proposed that this link between the Golgi complex and the CB plays a role in the accumulation of enzymes that will act in the nucleus posterior region during later spermiogenesis. The relationship between the CB and the Golgi complex may also be connected to the aggregative characteristics of the CB, which acts as a site where unnecessary DNA, RNA and proteins are degraded (Haraguchi et al. 2005).

Earlier spermatids were shown to have an association between the CB and the mitochondrias, and also an association between the CB and the lipid drops around the acrosomal vesicle formation region. These lipid drops are often in the germ cells of the species being studied herein. The same association between the CB and the lipid drops was observed in later spermatids of *Meriones unguiculatus* (Peruquetti et al. 2010a), which suggested that it may be involved in hormone metabolism during spermiogenesis. In this study, the connections between the CB, mitochondrial clusters, and lipid drops were also observed in the nuclear posterior region of later spermatids, where the spermatozoon tail and middle piece are formed. The CB has proven to be highly mobile in the germ cell cytoplasm, and this mobility may be related to the presence of actin (Walt and Armbruster 1984; Aumüller and Seitz 1988) and/or calcium (Andonov and Chaldakov 1989). Our results showed the CB in several different positions in the cytoplasm of the primary spermatocytes, earlier spermatids, and later spermatids. This high mobility was also reported by Parvinen et al. (1997) in living early spermatids of rats, which had CBs in rapidly changing positions in relation to the nuclear envelope, Golgi complex, and nuclear pale chromatin areas. Ribonucleoprotein material from the CB, lipid drops, and some mitochondria were observed in the residual bodies of later spermatids, indicating that these materials are eliminated and possibly digested at the end of the spermiogenesis process (Sud 1961; Yokota 2008).

In conclusion, our data show that the fragmentation of the nucleolus of primary spermatocytes occurs at the pachytene stage in the spermatogenesis of *Phrynops geoffroanus*. This process occurs at the same

time as when the CB starts its formation process. Thus, the nucleolus fragmentation may be involved in the formation of this germ cell structure, which seems to play important roles in various parts of the spermatogenesis process, such as the acrosome formation, as well as the formation of the spermatozoon tail and middle piece.

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