International Scholars Journals

International Journal of Animal Breeding and Genetics Vol. 6 (12), pp. 001-006, December, 2017. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

A comparative study of the gross structure and distribution of spermatogonia-like cells in the testis of pre-pubertal and mature adult rats

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Accepted 18 September, 2017

The testis of pre-pubertal and those of mature adult albino rats were physically divided into three segments with a view to studying histologically the gross structure and distribution of spermatogonia-derived cells (spermatogonia, spermatocytes, spermatids and mature spermatozoa) in the seminiferous tubules in these segments. After using 50% Mezo stain (organic), spermatogonia-derived cells were manually counted on slide-view. A mixed population of dense uninucleated single or paired type A-like spermatogonia was clearly identifiable in both upper and lower segments as well as in the middle segment of the testis of pre-pubertal rats. In the mature adult rat testis, a mixed population of spermatocytes, oval spermatids and mature spermatozoa, in addition to a mixed population of dense uninucleated and multinucleated type A- like spermatogonia were identified in seminiferous tubules of all three segments. However, there were larger numbers of clump-forming spermatogonia-like cells in the middle segment of the testis than in other segments in mature adult rats. A ratio of 2:1:1 for middle, upper and lower segments respectively was manually determined. These results suggest that the middle segment of the mature adult albino rat testis contains a highly enriched population of both single and multinucleated A-type spermatogonia-like cells and may be most suitable site for the harvesting of these cells.

Key words: Rat, pre-pubertal, testis, spermatogonia, spermatogenesis, mezo stain.

INTRODUCTION

There are about 0.03% of all germ cells that are stem cells in the adult mouse testes (Tegelenbosch and de Rooij, 1993) and only 3% of the type A spermatogonia in these adult mouse testis are stem cells (Tegelenbosch and de Rooij, 1993; de Rooij and van Pelt, 2003). These testicular spermatogonial stem cells renew themselves and still differentiate into oval or elongate spermatids and finally into spermatozoa (see review de Rooij and van Pelt, 2003; Kubota and Brinster, 2006) that are released into the lumen of the seminiferous tubules. This whole process initiates and sustains spermatogenesis through-out life. Spermatogonial stem cells mostly occupy a stem cell niche around the basement membrane (Chiarini-Garcia et al., 2001) the hormonal integrity of which (or transplantation of somatic elements into it) is required to restore the ability to support spermatogenesis (Zhang et al., 2006, 2007).

The physiological importance of the stem cell niche/ microenvironment has been suggested to be dependent on beta1-integrin as an essential adhesion receptor for spermatogonial stem cell homing (Kanatsu-Shinohara et al., 2008) and to be related to an ability or inability to support appropriate balance between self renewal and differentiation (Ryu et al., 2003, 2006), the basic function of spermatogonial stem cells. Spermato-gonial cell cohort can also switch on an apoptosis program in a coordinated fashion following forced contraction of Sertoli cells (Tres

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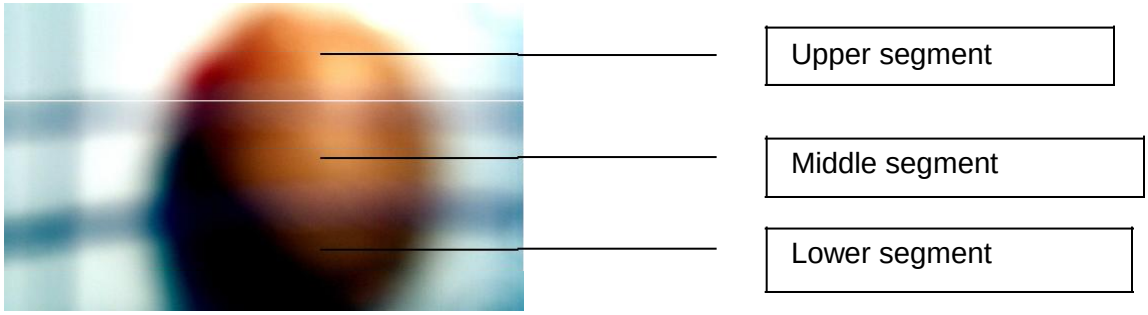

Figure 1. Photograph of the rat testis showing the physical segmentation into three (3) during our studies.

and Kierszenbaum, 1999) . Thus, it would be expected that a higher availability of different spermatogonial stem cell types in sufficient numbers in any one segment of the testis will enhance the possibility of spermatogonial stem cells being accessed in adult mouse or rat testicular tissue.

One of the difficulties encountered in studying regulation of spermatogonial stem cell self-renewal and differentiation in mature adult rats seems to be their localization and subsequent harvesting (de Rooij and van Pelt, 2003). Therefore, localizing a testicular region of a high population of both single and multinucleated type A spermatogonia-like cells in the mature adult rat testis will be desirable. An identified site may be usefully adopted for the harvesting of rat testicular type A spermatogonia-like cells during studies involving the use of these cell types.

If testicular segments containing unusually large populations of type A spermatogonia-like cells possessing the capacity to differentiate (e.g. presence of multinucleated cells) can be identified then cell occupants of this site can be harvested from such segments of cellular abundance and used in xenotransplantation experiments (Ogawa et al., 1997; Brinster and Nagano, 1998) for studying the molecular regulatory mechanisms in immuno- and fertility-suppressed (using busulfan or cyclosporine) rats undergoing recolonization by transfected stem cells (Orwig et al., 2002; Zhang et al., 2003).

We therefore reasoned that segmenting the whole length of the testis into three and studying the histology and structure of the spatial distribution of testicular cells in these segments may reveal potential gross sites for the harvesting of type A-like spermatogonial stem cells.

MATERIALS AND METHODS

Preparation of modified RPMI 1640 culture medium for rat testicular cells

Rosewell Park Memorial Institute (RPMI) 1640 MEDIUM HEPES Modification is a basic cell culture medium produced from ingredients of animal-free origin, used for a wide range of applications and adaptable for the optimal culture of many cell types (Literature insert, SIGMA-ALDRICH Inc., St. Louis, MO., USA.). This culture medium was further modified by the addition of gentamycin (1% v/v), a broad spectrum antibiotic to minimize microbial growth during incubation at 37^{o}C, fetal calf serum (10% v/v), that is mostly required to facilitate stem cellular activity and fetal bovine serum (10% v/v; SIGMA, USA). Double glass-distilled water was used wherever necessary.

Preparation of testicular cells for histologic examination

Prepubertal male rats weighing 65 - 95 g or mature adult male rats 200 - 300g body weight were anaesthetized under light ether anaesthesia for 3 - 7 min during which their scrotal sacs were swabbed using 70% ethanol in water and cut open to expose the seminiferous tubules. These were carefully dissected out and separated from blood vessels using a sterile scalpel and then immersed into the modified Rosewell Park Memorial Institute (RPMI) 1640 culture medium (Literature insert, SIGMA-ALDRICH Inc., St. Louis, MO., USA.). Tissue sample was placed on glass slide and stained with 50% Mezo (organic stain) for 15mins and then decolorised with 70% ethanol in water. Glass slide was subsequently rinsed with distilled water and air-dried for 10 - 15mins. Tissue sample on glass slide was manually examined under a light microscope with X40 or X100 (oil immersion) eye piece interfaced with a camera and computer output for higher magnification and better resolution. Single cells or cell clusters were clearly identifiable in each view of the slide where they are present. Cell content of the slide-view was manually counted. The photomicrographs were digitally stored or printed out on glossy photographic paper.

Grouping of testicular segments

Dissected out left or right testis was segmented into three (upper, middle and lower; Figure 1) and these segments were used for the studies. Cell suspension from each of these segments was mounted on the glass slide where the cells were clearly identifiable under the microscope and these were manually counted on the slide-view. The cell number estimate in the upper, middle and lower segments were compared with each other and used as observations representative of seminiferous tubular cellular content.

RESULTS

In all three segments (upper, middle and lower) of testicular (Figure 1) tissue samples taken from the seminiferous tubules, a mixed population of dense uninucleated single or paired type A-like spermatogonia was evenly observed in pre-pubertal rats (Figure 2). In the mature adult rat tes-

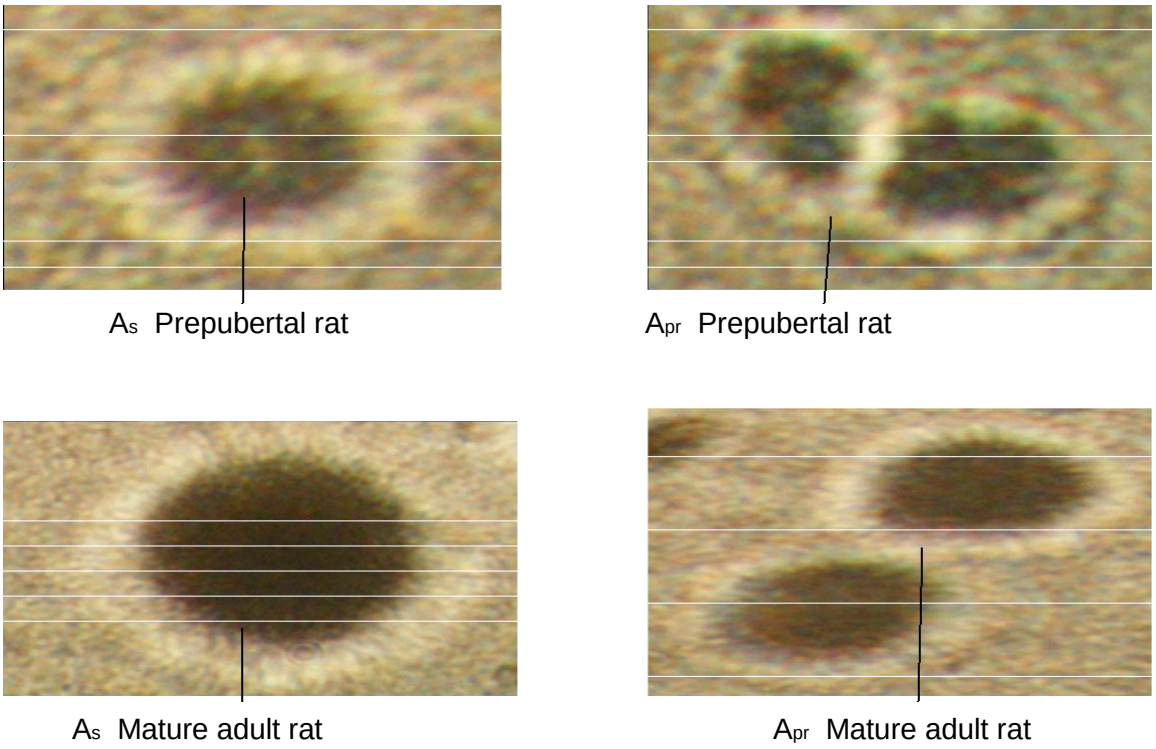

Figure 2. Photomicrograph of testicular A-type spermatogonia-like single or paired uninucleated cells from pre-pubertal rat or from mature adult rat stained using 50% Mezo Stain. A_s and A_{pr} represent single and paired spermatogonia-like cells respectively. These uninucleated A-type spermatogonia-like cells were found in all three segments of the testis. Tissue was stained using Mezo (organic) stain.

ticular tissue samples taken from the seminiferous tubules, a mixed population of spermatocytes, oval spermatids and mature spermatozoa were also observed in addition to a mixed population of dense uninucleated and multinucleated type A-like spermatogonia in all three segments (Figures 3 and 5). However, there were larger numbers of clump-forming spermatogonia-like cells in the middle segment of the testis of both prepubertal and mature adult rats (Figure 4) when compared with observations in both upper and lower segments which serve for comparative purposes.

Thus, tissue samples taken from the seminiferous tubules in the mature adult rat testis contain all the male germline cells observed in tissue samples taken from the seminiferous tubules in the pre-pubertal rats. Additionally, these tissue samples taken from the seminiferous tubules in the mature adult rat testis contain mature spermatozoa. The absence of mature spermatozoa in all segments of testicular tissue taken from prepubertal rats was conspicuous. A major observation on the distribution of germline cells in these three physical segments of the rat testis was the existence of large numbers of clump-forming A-type spermatogonia-like cells in the middle segment of the testis of mature adult rats. These larger numbers of clump-forming cells contrast with observations in both upper and lower segments which serve for comparative purposes.

DISCUSSION

The results of our studies show that a mixed population of dense uninucleated and multinucleated type A-like spermatogonia were clearly identifiable in the seminiferous tubules of both upper and lower segments as well as in the middle segment of the testis of mature adult rats. The identifiable cells are structurally similar to those in the spermatogonia generations earlier reported (Huckins and Oakberg, 1978). In their study of spermatogonial populations in normal adult mice, Huckins and Oakberg (1978) reported that undifferentiated A spermatogonia and their six generations of differentiating spermatogonia were clearly identifiable on whole mounts of seminiferous tubules and that these cell types behaved in essentially the same manner as their counterparts in the rat (Huckins and Oakberg, 1978). These differentiating progeny of spermatogonia were linked by cytoplasmic bridges (or intercellular bridges) forming mixed populations of these cell types (Huckins, 1978; Huckins and Oakberg, 1978). The results of our studies in rats are therefore in agreement with the observations in normal adult mice earlier reported (Huckins and Oakberg, 1978)

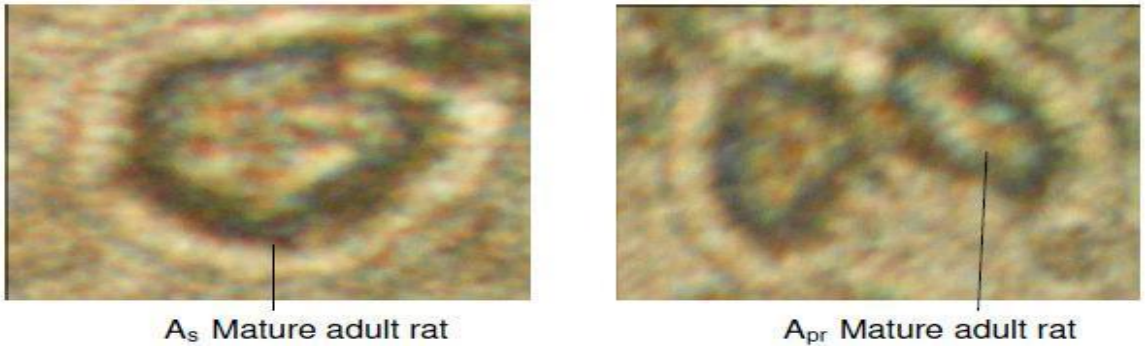

Figure 3. Photomicrograph of testicular spermatogonia-derived single or paired multinucleated cells from mature adult rat stained using 50% Mezo Stain. A_s and A_{pr} represent single and paired spermatogonia-like cells respectively. The multinucleated A-type spermatogonia-derived cells were found in all three segments of the testis. Tissue was stained using Mezo (organic) stain.

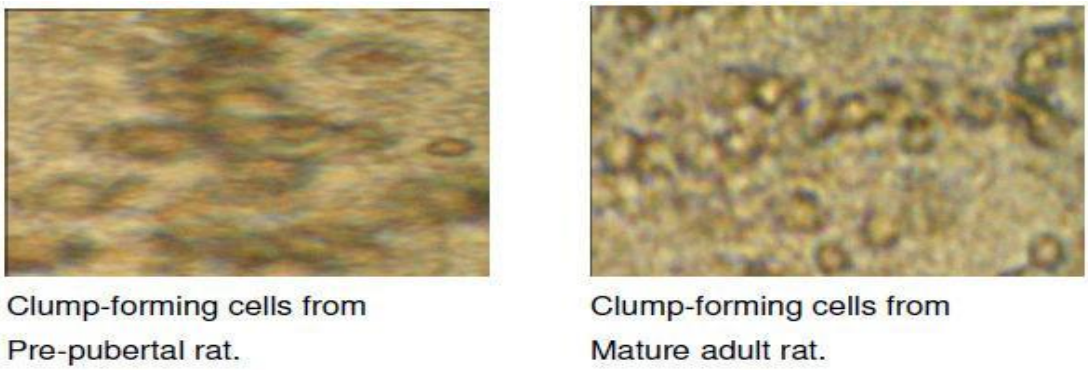

Figure 4. Photomicrograph showing clump-forming testicular A-type spermatogonia from pre-pubertal rat or larger numbers from mature adult rat stained using 50% Mezo Stain. These clump-forming testicular spermatogonia were found in all three segments of the testis from both pre-pubertal and mature adult rat testis. There were however, larger numbers of these clump-forming A-type spermatogonial cells in the seminiferous tubules of the middle segment of the mature adult rat testis.

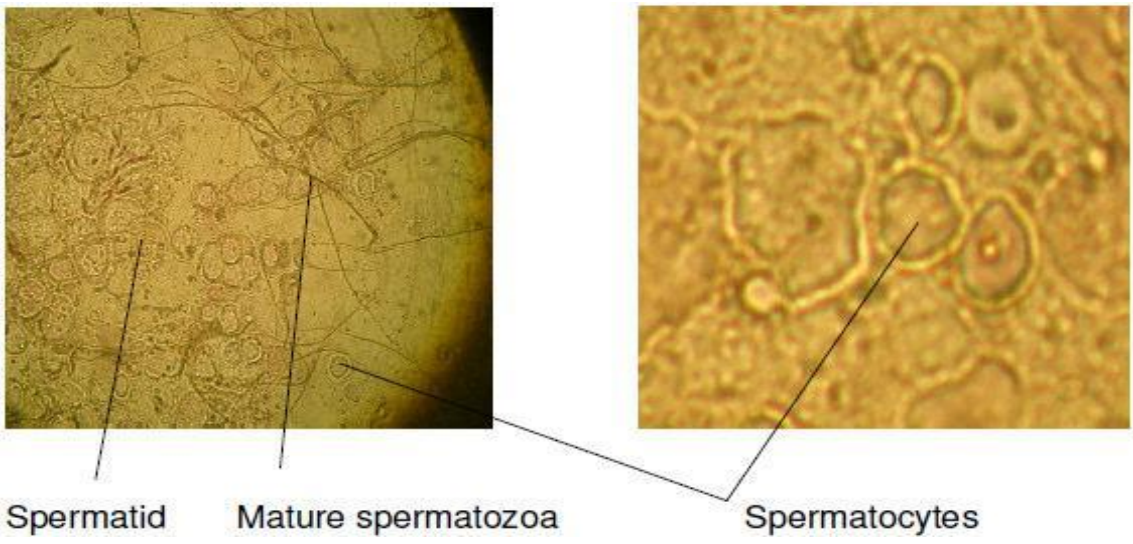

Figure 5. Photomicrograph showing testicular spermatocytes and oval spermatids from mature adult rat stained using 50% Mezo Stain. Spermatocytes and oval spermatids were found in all three segments of the testis in mature adult rats.

and our results further demonstrate that carefully searching these cell populations may be relevant to spermatogonial stem cell identification for research.

Coexistence of uninucleated and multinucleated spermatogonial cell types may have resulted from the formation of complex syncytial networks of progeny of paired type A and production of type B spermatogonia linked by cytoplasmic bridges (Huckins, 1978). The mixed populations of testicular germ cells are therefore characteristic of rodent testicular tissue where spermatogonial cell types exist in functional harmony with other somatic cells like Sertoli and Leydig cells. It can then be imagined that in a normal sample of seminiferous tubular cell suspension, these various cell types will be present. Our observation on the presence of a mixed population of undifferentiated and differentiating germline stem cells and spermatogonial progenitors in adult rat testicular cell suspension is consistent with earlier reports (Huckins, 1978; Huckins and Oakberg, 1978).

This observation of a mixed population of spermatocytes, oval spermatids and mature spermatozoa in both upper and lower segments as well as in the middle segment of the testis of mature adult rats is in agreement with the observation that testicular cell suspension is a mixed population of undifferentiated and differentiating germinal stem cells and spermatogonial progenitors in adult mice (Ogawa et al., 1997) and that microinjection of cell suspensions into the seminiferous tubules, efferent ducts or rete testis are equally effective in generating donor- cell derived spermatogenesis in recipients (Ogawa et al., 1997). The consequent spermatozoa represent fully differentiated germ cells having started from single spermatogonial stem cells. In the mature adult rat testis, the mixed population of spermatocytes, oval spermatids and mature spermatozoa observed in all three segments cannot be used as a distinguishing factor between our three physical segments as we could not identify unusually large numbers of these cell types in any of the segments.

Germ cell differentiation takes place in the seminiferous tubules of the testis when a population of diploid stem-cell spermatogonia that lie on the basement membrane of the tubule continuously undergoes self-renewal and produces progeny cells, which initiate the process of cellular differentiation to generate mature spermatozoa (Ogawa et al., 1997). Although several factors have been identified to participate in deciding the fate of a single spermatogonial stem cell (that is whether self-renewal, proliferation or differentiation), there is a temporal difference in proliferative activity that gives rise to a large variation in stem cell density between areas of the seminiferous epithelium (De Rooij, 1988). Thus, the observed mixed population of spermatophytes and oval spermatids in our studies may have originated from cellular differentiation of progeny cells to generate spermatids and spermatozoa in the mature adult rat testis.

However, there were larger numbers of clump-forming germline cells in the seminiferous tubules in the middle segment than in other segments of the testis of mature adult rats. Light microscopic examination of rodent testis revealed the presence of intercellular bridges in all classes of spermatogonia except for new A_S (single spermatogonial) stem cells and it was thought that these intercellular bridges mediated both differentiation and degeneration of spermatogonia (Huckins, 1998) . Clump formation among non-A_S spermatogonial cells will be facilitated by the formation of these cytoplasmic bridges. It is possible that the large numbers of clump-forming germline cells observed in the seminiferous tubules in the middle segment of the testis of mature adult rats during our studies would have resulted from large numbers of non-A_S spermatogonial cells.

Recently, it was suggested that culture medium conditioned by an artificial fibroblast line (SNL-CM), in the absence of somatic cells, is able to stimulate primary cultures of rat type-A single spermatogonia to develop into chains of aligned spermatogonia at the 8-, 16-, and 32-cell stages thereby giving rise to the identification of neuregulin as a factor required for formation of aligned spermatogonia (Hamra et al., 2007). An exogenous soluble factor that promotes proliferation of spermatogonial stem cells has also been identified as glial cell line-derived neurotrophic factor (GDNF) (Kubota et al., 2004). These factors caused formation of densely packed clumps of cells which continuously proliferated and were indeed identified as spermatogonial stem cells (Kubota et al., 2004). It is however, not clear whether these natural factors that can stimulate type-A spermatogonia to develop into clump-forming germline cells were present in our culture medium or whether there were larger quantities of these factors in the middle segment of the testis of mature adult rats.

It is also possible that our observation of these large numbers of cluster-forming spermatogonia-like cells in the seminiferous tubules taken from the middle segment of the testis does not represent a true anatomical difference since seminiferous tubular epithelial activities can be argued to be common along the whole length of the seminiferous tubules irrespective of which segments they may be in. However, a physical examination of the testis will show that, on the assumption that seminiferous tubular distribution is even, the greater physical bulk of such materials in the middle segment of our division will naturally contain a higher proportion of seminiferous tubules and consequently higher seminiferous tubular epithelial activities thus explaining our observation of larger numbers of cluster-forming germline cells in the middle segment.

A possible benefit of the results of this study is the observed existence of a highly enriched population of both single and multinucleated types of spermatogonia-like cells in the middle segment of the mature adult rat testis. This site may be preferred for the harvesting of rat testicular spermatogonia-like cells. This testicular site can

also serve for preferred transplantation site in immunosuppressed (using busulfan or cyclosporine) rats undergoing recolonization by transfected stem cells (Orwig et al., 2002; Zhang et al., 2003).

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