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Isolation and Molecular Characterization of Spermatogonial Stem Cells in Nile Tilapia

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Abstract

The identification and isolation of spermatogonial stem cells in Nile tilapia is crucial to the development of high-quality transplantation techniques as well as to understand the regulation of spermatogonia *in vivo*. However, specific molecular markers for spermatogonial stem cells have not yet been studied in detail. Taking advantage of this species as a good experimental fish model, we investigated the isolation and culture of spermatogonial stem cells from tilapia testis. Also, the expression of Gfra1, Notch1, and CD44 as molecular markers were studied using real-time RT-PCR, immunohistochemistry, and flow cytometry analysis. The result showed that enzymatic digestion, percoll gradient, and differential adhesion are suitable for the isolation of an enriched population of spermatogonial stem cells from tilapia´s testis. Moreover, the isolated spermatogonia expressed Gfra1, Notch1, and CD44 *in vivo* and *in vitro*. In conclusion, Gfra1, Notch1, and CD44 can be considered as good markers for spermatogonial stem cells in fish which may be effective to characterize and isolate spermatogonial stem cells from the Nile tilapia.

Key words: CD44, Flow cytometry, Gfra1, Notch1, real-time RT-PCR, SSCs.

1. Introduction

Nile tilapia is the most economically important freshwater fish species in Egypt. Tilapia is characterized by a fast growth rate, oral parental care, convenient size, and tolerance to low levels of dissolved oxygen **(Trewavas, 1984; Stickney, 2000)**. Besides, tilapia has the adaptation ability to a wide range of environmental and management conditions **(Silva, 1987; Stickney, 2000)**. These advantages make tilapia an excellent experimental model for investigating the biology of reproduction and gene expression studies among teleost fish species under laboratory conditions **(Stickney, 2000).**

Spermatogenesis is an extended, complex, cyclical process that is highly organized and coordinated (Nayak *et al.*, 2016). It begins with the proliferation of diploid spermatogonia and proceeds through the extensive morphological changes that are leading to the formation of a haploid mature functional spermatozoon **(Nóbrega *et al.*, 2009; Nóbrega *et al.*, 2010; Lacerda *et al.*, 2014)**. According to the morphological appearance and the number of germ cells per spermatocyst, spermatogonial generations in fish were classified as undifferentiated type A spermatogonia (Aund), differentiated type A spermatogonia (Adiff), and type B spermatogonia (B early and B late) **(Schulz *et al.*, 2010; Lacerda *et al.*, 2014)**. Continuous formation of spermatozoa throughout life in adult male vertebrates depends on spermatogonial stem cells (SSCs) that are a subpopulation of type A spermatogonia. These cells are characterized by the ability of self-renewal and differentiation **(de Rooij and Russell, 2000; Schulz *et al.*, 2010; Lacerda *et al.*, 2014)**.

In fish, Aund spermatogonia are presented as the actual SSCs. On the other hand, Adiff spermatogonia are thought to be a reserve of stem cells that restore stemness potency after clonal fragmentation **(Nagano and Yeh, 2013; Lacerda *et al.*, 2014)**. Although the stage of no return to stemness is still unclear, it is well established that type B spermatogonia are committed to differentiation in both fish and mammals **(Nagano and Yeh, 2013)**.

Spermatogonial transplantation has been established as a powerful technique utilized to investigate SSCs biology developed in the past decade **(Brinster and Zimmermann, 1994)**. Successful spermatogonial transplantation has been achieved in Zebrafish, Nile tilapia, and rainbow trout **(Ciruna *et al.*, 2002; Lacerda *et al.*, 2006; Okutsu *et al.*, 2007)**. These experiments allow great potential for studies including surrogate broodstock technology **(Yoshizaki *et al.*, 2012; Lacerda *et al.*, 2013)**. Therefore, the isolation and identification of SSCs are critical for improving the efficiency of spermatogonial transplantation and understanding their regulation *in vivo*.

Although, there are no unique molecular markers to identify SSCs in both fish and mammals **(Tegelenbosch and de Rooij, 1993)**, several potential spermatogonial cells´ markers have been investigated in fish, e.g. spermatogonia-specific antigen-1 (Sgsa-1), neurogenic locus notch homolog protein 1-lik (Notch1), POU domain class 5, transcription factor 1-like (Pou5f1), promyelocytic leukemia zinc finger (Plzf), GDNF family receptor alpha-1-like (Gfra1) **(Kobayashi *et al.*, 1998; Yano *et al.*, 2009; Nagasawa *et al.*, 2010; Sánchez-Sánchez *et al.*, 2010; Ozaki *et al.*, 2011; Lacerda *et al.*, 2013)**. In the undifferentiated spermatogonia, the Gfra1 receptor is needed for the

association of Glial cell line-derived neurotrophic factor (GDNF) with RET tyrosine kinase **(Trupp *et al.*, 1998)**. Gfra1 is the first studied molecular marker to distinguish undifferentiated type A spermatogonia from differentiated spermatogonia in fish **(Nagano *et al.*, 2003; Lacerda *et al.*, 2014; Wei *et al.*, 2017)**. The expression of the Gfra1 receptor has been observed in Aund spermatogonia of dogfish **(Bosseboeuf *et al.*, 2014),** rainbow trout **(Bellache *et al.*, 2014; Nakajima *et al.*, 2014),** and Nile-tilapia **(Lacerda *et al.*, 2013)**.

Notch1 protein function in the maintenance of the proliferation of spermatogonial stem cells **(Lacerda *et al.*, 2014*;* Crespo *et al.*, 2016)**. **Hayashi et al. (2004)** suggested that Notch1 is involved in spermatogonial stem cell proliferation as well as differentiation. By using Transcription polymerase chain reaction (RT-PCR), the expression of Notch1 protein has been studied in trout **(Yano *et al.*, 2009).**

CD44 or homing cell adhesion molecule (HCAM) functions in adhesion, migration, homing, proliferation, and apoptosis of stem cells **(Knudson and Loeser, 2002)**. Also, CD44 has a role in stemness maintenance by involving contact between stem cells/progenitor cells and their cellular niche **(Zhu *et al.*, 2006)**. **Zheng et al. (2013)** have detected the expression of protein CD44 protein in spermatogonial stem cells of mice by RT-PCR and immunohistochemistry.

Experimental investigation of type A spermatogonium has been limited, and little is known about the biology of SSC especially in fish due to the limited availability of specific markers, their few numbers, and their physiological properties are still poorly understood. For these reasons, we aimed by this work to isolate, culture, and investigate SSCs markers from tilapia's testes. For this, we applied the real-time RT-PCR, flow cytometry, and histochemical methods to investigate the expression of Gfra1, Notch1, and CD44 by these cells.

2. Materials & Methods

2.1. Experimental animals

A total of 53 mature male Nile tilapia (*Oreochromis niloticus*) were used in this investigation. The selected tilapias were obtained from a fish farm belonging to the faculty of agriculture of Kafr El-Sheikh governorate at the age of 90 days, with a weight of 130 g and a total length of 14 cm. They were immediately transported to the laboratory in plastic containers containing dechlorinated tap water. They were kept in the laboratory in a closed recirculating water system in plastic tanks containing 250 liters of water at pH of 7.3, the temperature of 25 ℃ in winter, the oxygen level of 7.5-8.0 mg/L with alternating 12 hours light and dark cycles (12D:12L) and were fed standard food pellets contain 50% protein and 16% fat.

2.2. Testes collection

Testes were aseptically collected from male tilapia in a sterilized dish containing Dulbecco's phosphate-buffered saline (DPBS) and were placed on ice. Testes were minced into small pieces (1-2 mm^3) and washed several times with Hank's Balanced Salt Solution (HBSS) containing streptomycin 100 mg/ml and penicillin 100 U/ml.

2.3. Isolation of type A spermatogonia

The protocols utilized to isolate type A spermatogonia in tilapia were modified from **Cytochem et al.'s (1977) and Chaves-Pozo et**

al.'s (2004) protocols. Thus, testicular fragments were enzymatically digested with collagenase (type I, Sigma Cat. No. DN25), and DNAse I (Sigma Cat. No. C9891) at 25 ℃ in an incubator. After this step, testicular tissue was resuspended in a solution of 0.25 % trypsin-EDTA at 25 ℃. After 30 minutes under gentle agitation, 10 % (v/v) of Fetal Bovine Serum (FBS) was added. The pellet of the undigested fragments was excluded by centrifugation. The supernatant was then processed by the consequent filtration process through a nylon filter with pores of approximately 60 μm. DNase I (20μg/mL) was added to facilitate the filtration process.

2.4. Purification

Cells obtained from enzymatic dissociation of testes were fractionated by discontinuous percoll gradient as previously described by **Lacerda et al. (2006)**. Four different concentrations were utilized to prepare the gradient being used: 10%, 25%, 30%, and 40%. Finally, the cell suspension was placed on the top of the percoll column and centrifuged for 30 min (800×g) at 25 ℃.

Based on the morphological characteristics of the cells, the two upper bands were selected due to their larger concentration of spermatogonia. After the cells were collected and washed, they were placed in DMEM/F12 supplemented with 10% FBS and cultured overnight with cell density adjusted to 3×10^6 cells. This procedure is the so-called differential plating adhesion **(Okutsu *et al.*, 2006)**. It is based on that somatic cells can adhere to the culture plate while the germ cells remain suspended in the supernatant or weakly adhered to the culture plate. The media containing non-adherent spermatogonia were removed gently. Sertoli cells detached using a solution of 0.25% trypsin/EDTA in DPBS diluted 1:10. Subsequently, the cell number and viability were detected by the trypan blue test.

2.5. Coculture of type A spermatogonia and Sertoli cells

The detached Sertoli cells were collected and seeded at a suitable concentration on 6-well plates containing DMEM)/F12 supplemented with epidermal growth factor (EGF) (10 μg/ml), 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin. Sertoli cells incubated at 25 ℃ for 3 hours to permit their attachments to the well plates. After that, the isolated type A spermatogonia from testes were added to the attached Sertoli cells. They were co-cultured for 5 days at 25 ℃, 5% CO_2, and the media were changed every two days. The cells´ numbers and viability were detected by the trypan blue test. Trypan blue 0.4% (1:1, v/v) was added to the cell suspension. After that, the number of dead and viable cells were obtained using a hemocytometer.

2.6. Real-time reverse transcription-polymerase chain reaction (PCR)

Total RNA from the isolated testes, cells of percoll fraction A and cultured spermatogonia were extracted using RNX kit standard (VIVANTIS Malaysia Cat. No. GF-TR-050) according to the manufacturer's guidance. The purity and integrity of RNA were examined by a 260/280 nm ratio measurement via a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Total RNA was treated with DNase I to remove genomic DNA. cDNA synthesis was achieved

using oligodT primers and superscript II reverse transcriptase. The primers specific for *gfra1, notch1, CD44,* and glyceraldehyde 3-phosphate dehydrogenase, testis-specific-like (*gadph*) genes as shown in **Table 1**. *gapdh* served as a reference gene **(Yano C.G. *et al.*, 2013)**. Real-time RT-PCR was accomplished using 1 µg cDNA, the primers, and PCR Supermix and Maxima SYBR Green qPCR Master Mix (2X) kit (Thermo Scientific). RT-PCR was carried out in triplicates under the following conditions: 95 ℃ for 10 minutes, followed by 40 cycles at 95 ℃ for 15 s, 58 ℃ for 30 s, and finally at 72℃ for 30 s. Reactions were run on a (PIKO REAL 96 Real-Time PCR system, Thermo Scientific). All values were normalized to the *gapdh* gene. The relative quantification ratio of gene expression was quantified using the $2^{\Delta\Delta CT}$ method **(Livac and Schmittgen 2001)**. PCR products were separated using 1.7 % agarose gel electrophoresis. The bands were visualized via Gel analyzer pro v 3.1 for automatic detection of bands and lanes.

2.7. Flowcytometry

According to the protocol described by **Brown and Wittwer (2000)**, the cell suspensions were prepared from testicular samples with Tris-EDTA buffer (pH 7.4) (Invitrogen.). The cell suspensions were fixed in 96-100 % ethanol (Invitrogen.) at 4 ℃ for 12 h, centrifuged at 1,500 rpm for 15 min, and then rinsed in PBS containing 50 µg/mL propidium iodide (PI) (Invitrogen.). For each sample, the analysis was based on the measurement of 10000 cells. Single-cell suspensions were prepared from testes from at least five tilapias, and 1.5-3 x 10^6 cells were stained for expression of Notch1-FTTC (Pharmingen) was submitted to cell sorting according to the expression of Notch1 using a FACS IV cell sorter (Becton Dickinson).

2.8. Immunohistochemistry

Immunohistochemistry was carried out to analyze the expression of Notch1 as described previously by **Syrbu and Cohen (2011)**. The testicular samples (n = 3) were fixed in 10 % buffered formalin (pH 7.4) for 24h, washed three times with PBS. After dehydration in an ascending series of alcohols, the testicular samples were embedded in paraffin at 60° C overnight, Serial sections of 5 µm in thickness were cut and dewaxed in xylene and rehydrated through a descending series of alcohols. Antigen retrieval was achieved by digestion in 0.05 % trypsin (pH 7.8) for 15 min at 37 ℃ and incubated with antibody against NOTCH1 (1:200), at 4℃ overnight after thoroughly mixing, followed with DAB to detect the immunoactivity and then Counterstained with Mayer hematoxylin. The primary antibody was replaced with PBS and used as a negative control. Specimens were observed with a light microscope and photographed.

2.9 Statistical analysis

Results were expressed as means SE. Statistical significance was analyzed by one-way analysis of variance (ANOVA). Statistical analysis was carried out using SPSS 17 software. Differences were considered statistically significant at $P < 0.05$.

3.Results

3.1. Isolation of type A spermatogonia

In comparison with control **(Fig. 1a), the**

Collagenase enzyme digested and degraded the collagen layers surrounding the tubule resulted in a loosening of the connective tissue elements and liberating the interstitial cells **(Fig. 1b)**. After collagenase digestion, the testicular tissue is treated with trypsin to digest extracellular matrix protein between epithelial cells to separate germ cells. The cytoplasmic bridges between germ cells were dissociated by the action of trypsin and by the subsequent pipetting. The germ cells appeared to repair rapidly any discontinuities in the plasma membrane of the cytoplasmic bridges **(Fig. 1c)**.

3.2. Purification of type A spermatogonia

The obtained cell suspension was collected and fractionated on a discontinuous percoll gradient into fractions. Quantitive analysis based on morphological criteria showed that fraction A with a percoll concentration of 10% contained most of type A spermatogonia **(Fig. 2a)**. Fraction B with a percoll concentration 30 contained type B spermatogonia and spermatocytes **(Fig. 2b)**. Fraction C with a percoll concentration of 40% contained somatic cells, spermatocytes, and germ cell aggregates **(Fig. 2c)**. The cells of fraction A of the gradient were plated on gelatin-coated 6-well plates **(Fig. 3a)**. Most of the somatic cells strongly adhered to this gelatin, while only a few types A spermatogonia were attached. Spermatogonial cells collected by pipetting **(Fig. 3b)**.

During the purification steps, Sertoli cells were with an irregular outline and appeared almost granular under an Inverted microscope **(Fig. 3a,3c)**. Type A spermatogonia observed were large spherical cells with large round nuclei contain eccentrical nucleoli **(Fig. 3b)**. Also, type B spermatogonia and early meiotic cells might be observed in the cell suspension.

3.3. Purity of type A spermatogonia

After enzymatic digestion, the yield of the seminiferous epithelial cells per 1 gram of testicular tissue was $1.44x10^7$ cells. The cell suspension contained on average 14.65% type A spermatogonia. percoll gradient separation and subsequent differential plating significantly increase the purity of these cells to 61.54% and 81.44%, respectively **(Fig. 4)**.

3.4. Coculture of type A spermatogonia

After purification, type A spermatogonia are cocultured with Sertoli cells as a feeder layer in DMEM/F12. Most of the somatic cells were attached to the plate, while only a few types A spermatogonia were attached **(Fig. 3a)**. At 24 hours after co-culture, most of the viable cells including type A spermatogonia were attached. Sertoli cells started to spread and form many different shapes. Type A spermatogonia were randomly dispersed on Sertoli cells layer were round in shape. Cytoplasmic processes between Sertoli cells and spermatogonia could be observed for the first time **(Fig. 5a)**.

At 48 hours after co-culture, round type A spermatogonia further developed into chains connected through an intercellular bridge **(Fig. 5b)**. At 72 hours of coculture, Sertoli cells and spermatogonia approached each other and form colonies. These colonies were noted in some areas of the culture plate and continued to grow during their development **(Fig. 5c-e)**.

3.5. Viability

The average rate of viability after enzyme digestion of testicular tissues and purification steps 85.44% and 78.50% respectively **(Fig. 4)**. When cells were cultured in vitro in DMEM, cell viability was reduced to 70.25% after 24 hours. The percentage of viable cells increased to 74.18, 75.01, and 75.64 after 2, 3, and 4 days, respectively. The percentage of viable cells at day 5 was declined to 72.55% **(Fig. 6)**.

3.6. Real-time Rt-PCR

RNA isolated and examined for purity, integrity. The absorbance ratio at 260/280 nm of all the samples was between 1.7 and 2.0, except for two samples with a ratio below 1.7, indicating most of the samples were free from contaminants. The size of RT-PCR product bands was confirmed by Agarose gel electrophoresis analysis **(Fig. 7a)**. The values obtained from real-time RT-PCR were normalized to the *gapdh* gene. Ct value for *gapdh* was between 19.79 and 22.95. Results analysis showed that cells from percoll fraction A demonstrated a high expression level of *gfra1, Notch1, CD44* compared with cells from the testes. Interestingly, the expression of *gfra1, Notch1, CD44* was significantly higher in cultured cells than the cells from percoll fraction A **(Fig. 7b)**.

3.7. Flow cytometry

Notch1 positive cells were detected using flow cytometry with an anti-Notch1 antibody. The results showed that the rate of Notch1 positive cells was 12.75% in the testicular samples. While in enriched type A spermatogonia the positive fraction was 83.58% **(Fig. 8)**.

3.8. Immunohistochemical analysis of testes and cultured spermatogonia

Notch1 expression is localized in the seminiferous tubules, in type A undifferentiated spermatogonia (Aund) and type A differentiated spermatogonia (Adiff). In contrast, type B spermatogonia, spermatocytes, spermatids, and spermatozoa, as well as testicular somatic cells did not show any expression for Notch1 **(Fig. 9)**. Also, immunocytochemical analysis of cultured spermatogonia using specific anti-Notch1, revealed that cultured type A spermatogonia express the Notch1 protein **(Fig. 10)**.

4. Discussion

Isolation and characterization of type A spermatogonia from tilapia could greatly contribute to the understanding of the mechanisms involved in the control of spermatogonial proliferation **(Morena *et al.*, 1996)**. However, isolation of type A spermatogonia is very difficult due to the presence of many stages of germ cells in the testes and the small number of type A spermatogonia **(Koruji *et al.*, 2012)**. The planned goal of that research was to improve a new technique for isolation and characterization of type A spermatogonia.

After selection of healthy testes of Nile tilapia, Type A spermatogonia were successfully isolated by enzymatic digestion and purified by percoll gradient and differential adhesion. The purified population of type A spermatogonium was successfully co-cultured with Sertoli cells for 5 days. Our results showed that Gfra1, Notch1, and CD44 can be considered as good markers for SSCs in tilapia. The results of this study pave the way for the in vitro study of differentiation, transplantation, and gene characterization of tilapia's SSCs.

During the last decade, Enzymatic digestion has been used to isolate type A spermatogonia from testis of several species including fish **(Nickkholgh *et al.*, 1999; Izadyar *et al.*, 2003; Lacerda *et al.*, 2006)**. Enzymatic digestion is designed to dissociate the testicular tissue into a monodisperse cell suspension, maximize cell yield, and give viable cells that maintain their morphological features **(Cytochem *et al.*, 1977)**.

Our investigation showed that enzymatic digestion was economic, simple, and effective for the isolation of monodisperse cell suspensions from the seminiferous tubules. In this experiment blowing a pipette helped in destroying the intracellular junctions between differentiating spermatogonia. This finding was similar to those of **Wu et al. (2009)** and **Lacerda et al. (2006),** who suggested that this method has a small negative effect on the viability of type A spermatogonia and may extend the time required for their attachment.

After enzymatic digestion, type A spermatogonia could be purified by purification methods such as percoll centrifugation and differential adhesion **(Zheng *et al.*, 2014).** In the current study, percoll centrifugation and differential adhesion were used for purification of type A spermatogonia because these techniques were easier, less costly, safer, faster, and had small negative effects on cell viability and their morphological features **(Heidari *et al.*, 2014)**. The maximum purity of type A spermatogonia obtained was 81.3% with high viability. Also, the results showed that purified cells exhibited normal morphological features and a high viability ratio. These results are consistent with the results obtained by **Wu et al. (2009)** and also, go following the previous studies of **Lacerda et al. (2006)** who applied percoll to separate type A spermatogonia in tilapia.

Due to the lack of nursing somatic cells and growth factors, spermatogonia depended on itself to achieve the process of attaching to the well plate **(Wu *et al.*, 2009)**. The conditions required for culturing of SSCs usually consist of two categories: one is to add growth factors such as BFGF, and GDNF to the media; or adding a feeder layer of Sertoli cells **(Liu *et al.*, 2011)**. In the current work, a feeder layer of Sertoli cells was used because of the high price of GDNF. The results reported that type A spermatogonia had successfully dispersed and grown on Sertoli cells.

The Sertoli feeder layer helps spermatogonia to withstand the external environment better. Moreover, type A spermatogonia had formed stable colonies for 5 days on Sertoli cells. This finding was similar to those reported by **Kiger et al. (2000) and Wu et al. (2009)** who have been declared that Sertoli cells can release all kinds of factors needed by spermatogonia growing in vitro. Also, the results in harmony with **Liu et al. (2011)** who have been reported that type A spermatogonia had a rapid adherence, marked proliferation and were able to form large numbers of colonies on the Sertoli feeder layer. Furthermore, **Lacerda et al. (2013)** found that type A spermatogonia cultured without Sertoli cells had a shorter life-span and a longer adhesion time when they were cultured in vitro.

The results showed that the viability of the cells was declined at 24 hours. This reduction may be due to the change in environmental settings besides the effect of enzymatic dissociation **(Kiger, *et al.*, 2000)**. The results also, revealed that after 48

hours, type A spermatogonia had proliferated and the viability had increased. These results in good agreement with data obtained by **Wu et al. (2009)** who have been suggested that cultured spermatogonia were able to adapt to the cultural environment and the other factors necessary for their growth. Our result showed that after a transient proliferative phase, the viability of type A spermatogonia began to decrease gradually. These findings were similar to those reported by **Liu et al. (2011)** who have been suggested that the decrease in the viability is due to the increasing deficiency in available growth factors such as glial cell line-derived neurotrophic factor (GDNF).

The lack of SSC molecular markers has been considered as a great limitation to identifying or purify SSCs in fish **(Hofmann *et al.*, 2005)**. Our study has investigated the expression of proteins (Gfra1, Notch1, CD44) which are considered to be markers of rodents SSCs in Nile tilapia testis **(Lacerda *et al.*, 2013)**. Gfra1 is the first studied molecular marker to characterize undifferentiated type A spermatogonia cells from differentiated spermatogonia in the teleost testis **(Lacerda *et al.*, 2013)**. The expression of the Gfra1 receptor has been observed in Aund spermatogonia of dogfish by RT-PCR and immunocytochemistry **(Bosseboeuf *et al.*, 2014)** and in rainbow trout by immunocytochemistry, RT-PCR, and flow cytometry **(Bellache *et al.*, 2014; Nakajima *et al.*, 2014)**. The expression of Gfra1 in the Nile-tilapia SSCs is similar to that of other fish and rodents **(Lacerda et al., 2013).** Our real-time RT-PCR results confirmed that type A spermatogonia express the protein Gfra1 in testes and during culture. Also, results showed that cells from percoll fraction A demonstrated a high expression level of Gfra1 compared with cells from the testes indicating a higher percentage of type A spermatogonia. Interestingly, the expression of *gfra1* was significantly higher in cultured cells may be due to their higher proliferation activity.

Also, expression of Gfra1 by cultured type A spermatogonia were significantly higher in comparison to testes, although the recombinant growth factor GDNF was not used as a supplement in the culture medium. These results may be due to the presence of Sertoli cells, which secrete GDNF in the medium. These observations are following the study of **Liu et al. (2011)** who have been demonstrated that Gfra1 is a good marker to characterize type A spermatogonia in tilapia.

Notch1 protein help in the maintenance of the proliferation of type A spermatogonia **(Nagano *et al.*, 2003)**. **Nagano et al. (2003)** reported that the expression of Notch1 in mouse testes stimulates the self-renewal of spermatogonial stem cells. Immunohistochemistry revealed that both undifferentiated and differentiated type A spermatogonia express the protein Notch1 in testes. Also, real-time RT-PCR results showed that the level of expression of Notch1 significantly increased during short-term culture. This result confirms the involvement of Notch1 in spermatogonial proliferation. These observations are following the study of **Yano et al. (2009)** who have been suggested that Notch1 is suitable for the isolation of type A spermatogonia from fish testes. Moreover, flow cytometric results showed that the percentage of Notch1 positive cells significantly increased which indicates the effectiveness of isolation and purification methods for isolation of SSCs.

CD44 protein has been considered that

functions in the proliferation of stem cells and have a role in stemness maintenance by involving in contact between stem cells/progenitor cells and their cellular niche **(Knudson and Loeser, 2002; Zhu *et al.*, 2006)**. **Schieker et al. (2004)** have been detected the expression of protein CD44 protein in mice by RT-PCR and immunohistochemistry. The real-time RT-PCR results confirmed that cultured type A spermatogonia express the protein CD44 during short-term culture. These observations are following the study of **Schieker *et al.* (2004), and Nagano and Yeh (2013)** who have been suggested that CD44 may play an important role in the proliferation of rat undifferentiated spermatogonia in the rat. Therefore, we suggest that CD44 can be employed as a molecular marker for the type A spermatogonia population in Nile tilapia or other fish species.

Conclusion

We found that the enzymatic procedure is an economical and reproducible method for isolation and purification of SSCs from fish. The establishment of coculture of SSCs with Sertoli cells had provided a useful cell model for studying the proliferation and SSCs markers. The results of this study pave the way for the in vitro study of proliferation, transplantation, and gene characterization of fish SSCs.

Moreover, investigation of spermatogonial physiology and their niche characteristics is a critical aspect for improving the use of transplantation strategy, and to apply various biotechnologies to preserve the germplasm. Moreover, it would be helpful for the formation of gene banks, improving commercial fish production, and the preservation of the genetic resources of endangered fish species in the future.

Annex 1: Figures and Figures´ captions

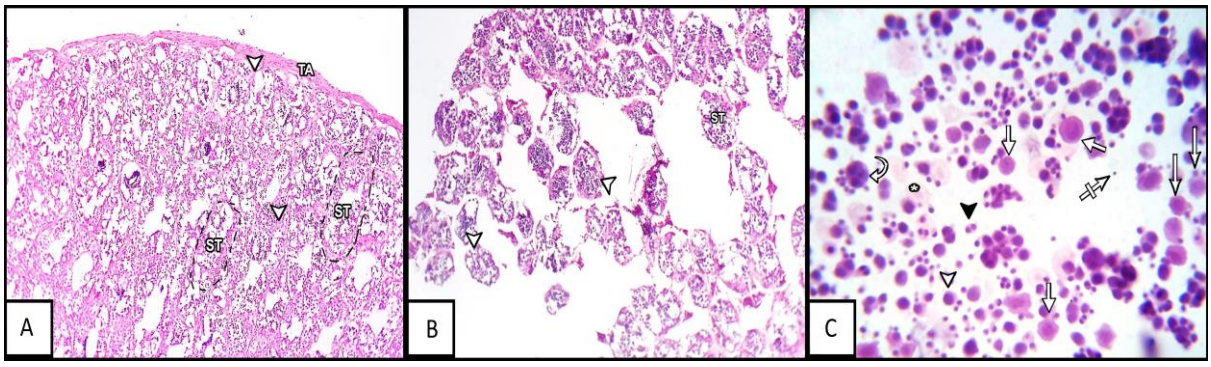
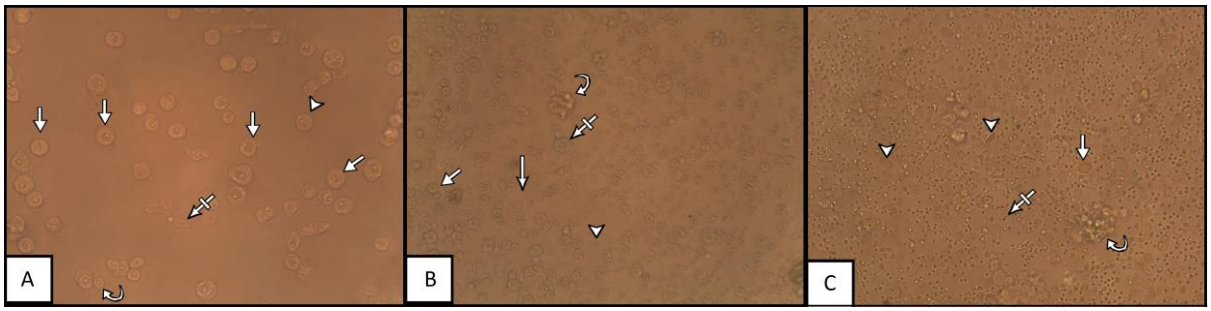
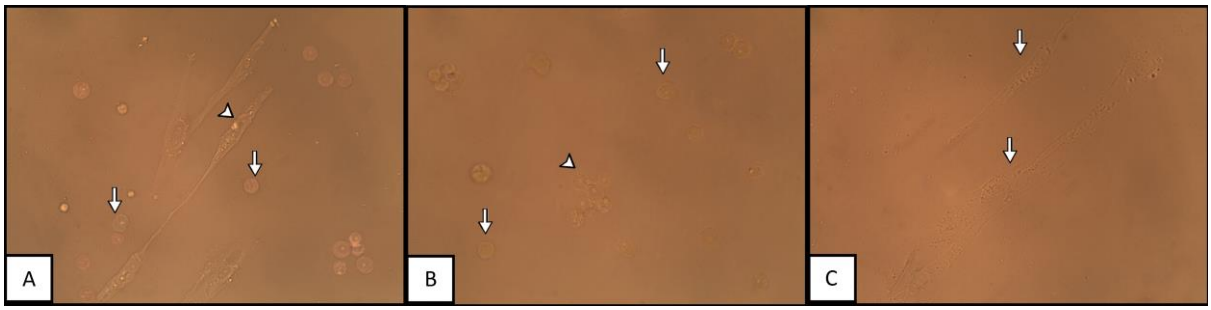


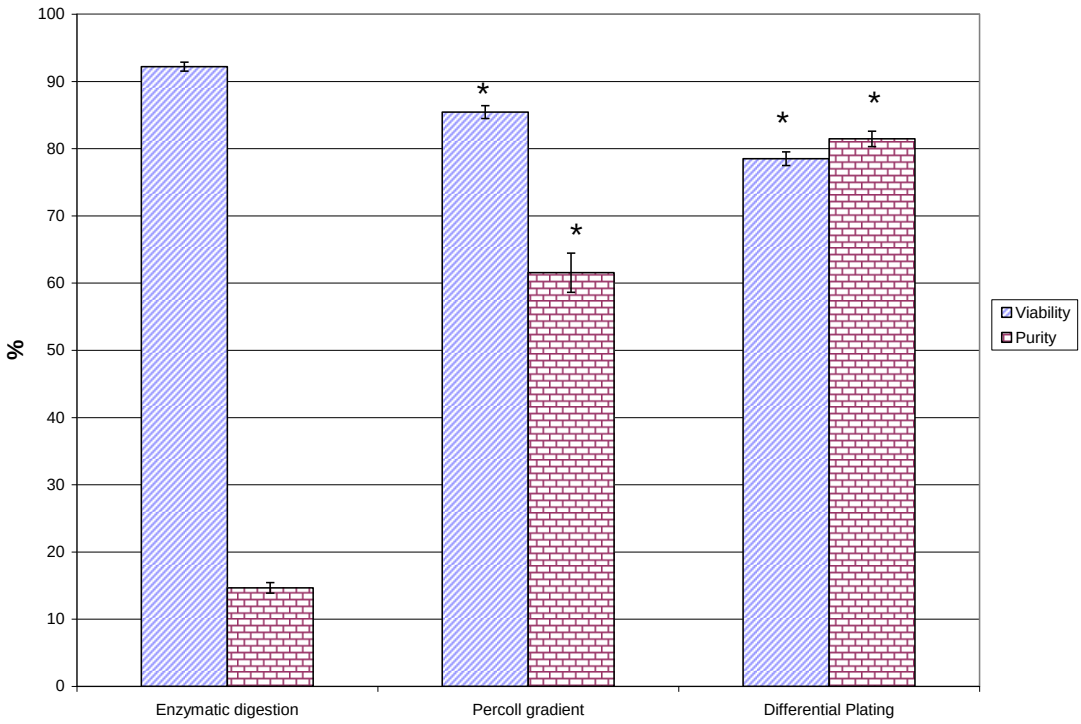
Fig. 1): A photomicrograph showing enzymatic dissociation (**A)** normal testis of Nile tilapia before dissociation (**B)** testicular tissue after collagenase digestion**.** Note; tunica albuginea **(TA);** seminiferous tubules **(ST)** surrounded by collagen fibers and interstitial tissue **(arrowhead) H&E X100. (C)** Cytospin preparation of cell suspension after digestion by trypsin showing; type A spermatogonia **(arrows)**, type B spermatogonia **(long arrows)**, spermatocyte **(white arrowheads)**, spermatids **(black arrowhead)**, spermatozoa **(cross arrow),** somatic cells **(star)** and a cluster of germ cells **(curved arrows)**. **H&E X400.**



(Fig. 2): A photomicrograph of percoll gradient centrifugation. **(A)** cells of percoll fraction A reveals a high percentage of type A spermatogonia **(arrows)**, type B spermatogonia **(arrowheads)**, spermatocyte **(curved arrows)**, somatic cells **(cross arrow)**. **X400 (C)** cells of percoll fraction B containing spermatocyte **(arrows)**, spermatids **(arrowheads)**, clusters of germ cells **(curved arrows)**, type B spermatogonia **(cross arrow),** and spermatozoa **(long arrows)**. **X400. (D)** cells of percoll fraction C showing spermatocytes **(arrows)**, spermatids **(cross arrow)**, red blood cells **(arrowhead),** and sperm were the predominant cells. **X100.**

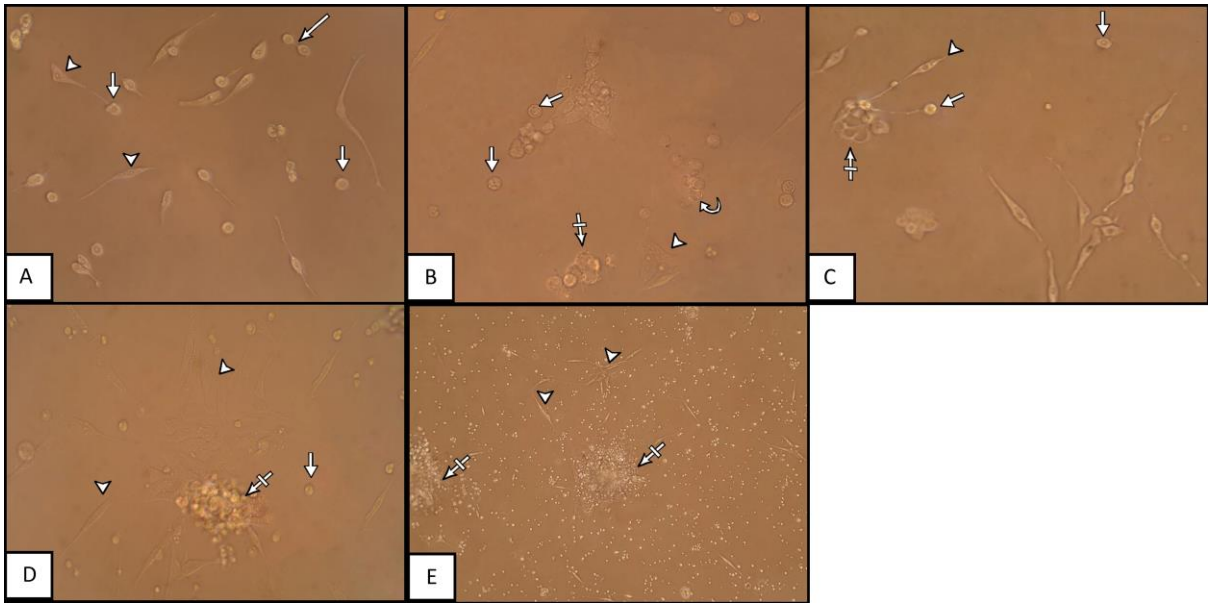


(Fig. 3): A photomicrograph of differential plating displaying: **(A)** cells cultured for 12 hours in gelatin-coated well-plates**;** type A spermatogonia **(arrows),** testicular somatic cells firmly attached to the culture plates **(arrowheads)**. **X400 (B)** Purified gelatin non-binding type A spermatogonia **(arrows),** clumping of spermatogonia **(arrowheads)** is due to the centrifugation procedure. **X200. (C)** Purified gelatin-binding Sertoli cells **(arrows)**. **X400**



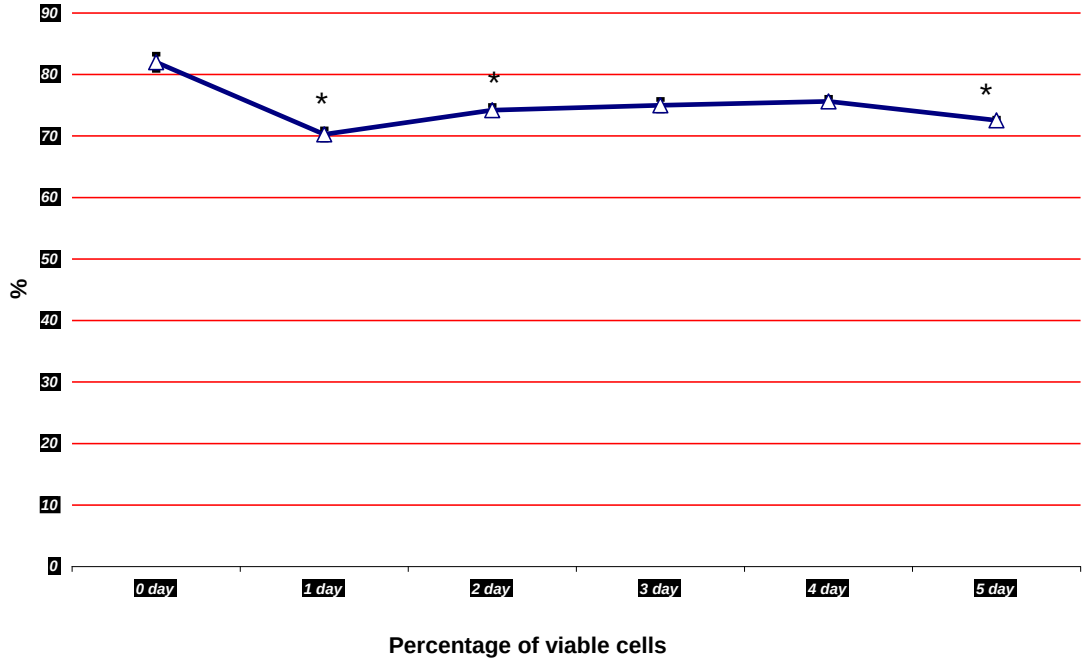
(Fig. 4):

Percentage and viability of type A spermatogonia. * significant at P < 0.05.

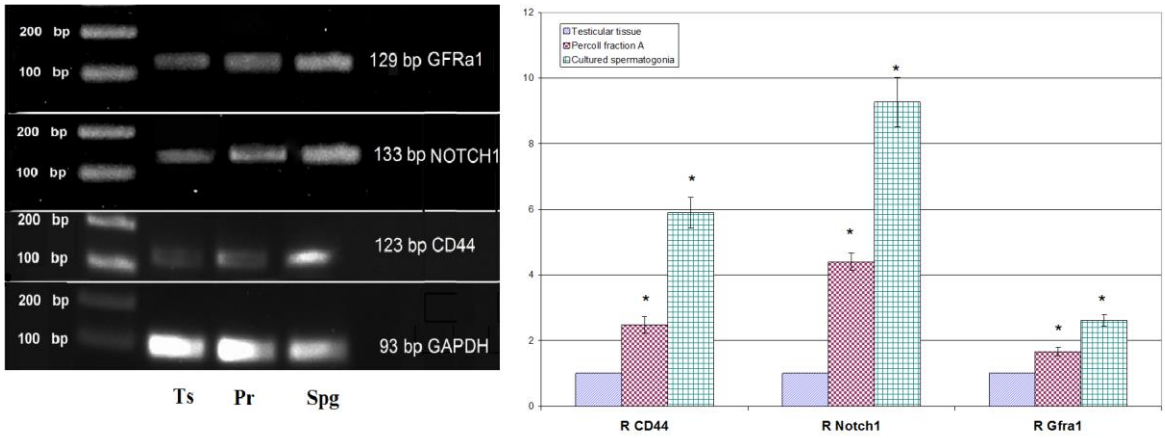


(Fig. 5): A photomicrograph of cultured type A spermatogonia. **(A)** after 24 hours. **X400**, **(B)** after 48 hours. **X400, (C)** after 72 hours. **X400**, **(D)** after 96 hours, **(E)** after 120 hours. **X200**. **Note;** Sertoli cells **(arrowheads),** type A spermatogonia **(arrows),** and intercellular bridges between germ cells **(long arrow)**. Colony type A spermatogonia **(cross arrow),** a chain of cultured type A spermatogonia

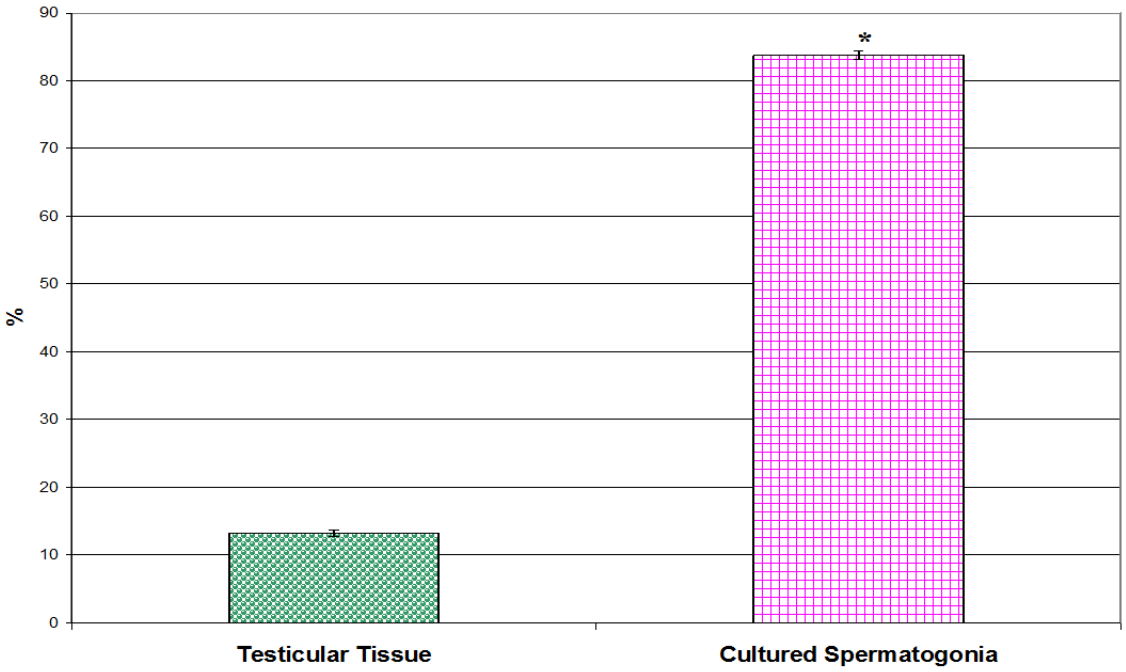
(curved arrow).



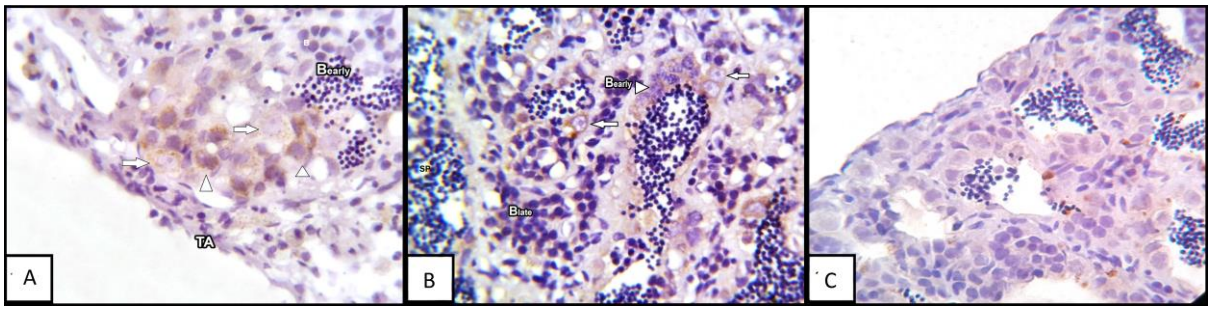
(Fig. 6): Viability of type A spermatogonia during coculture. * significant at P < 0.05.



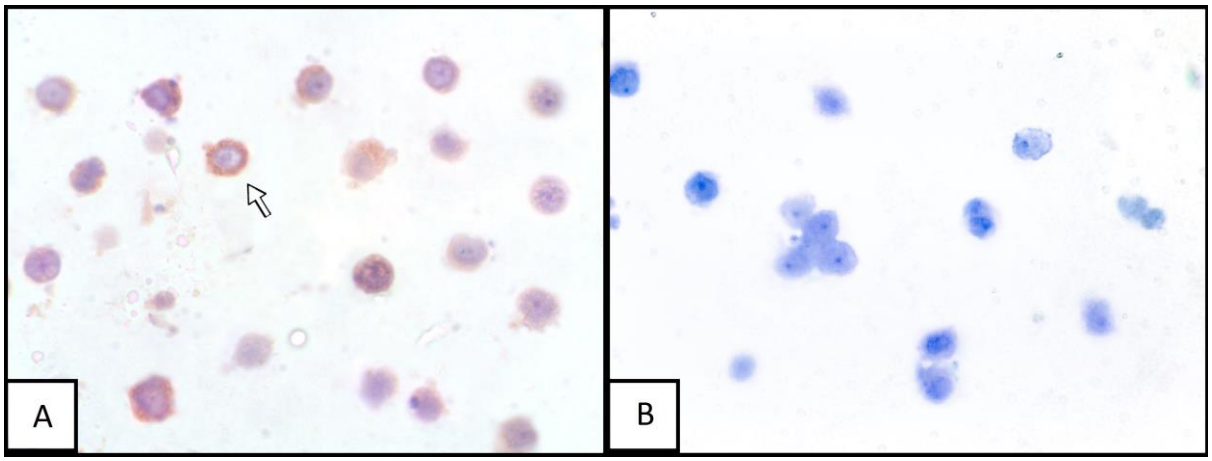
(Fig. 7): (A) A photomicrograph of the gel showing: expression of *gfr1, notch1, CD44* and *gapdh* (control gene) in testicular tissue **(Ts),** percoll fraction A **(Pr)**, and Cultured type A spermatogonia **(Spg).** Relative expression of *gfr1, notch1, and CD44* normalized by gapdh. * significant at P < 0.05.



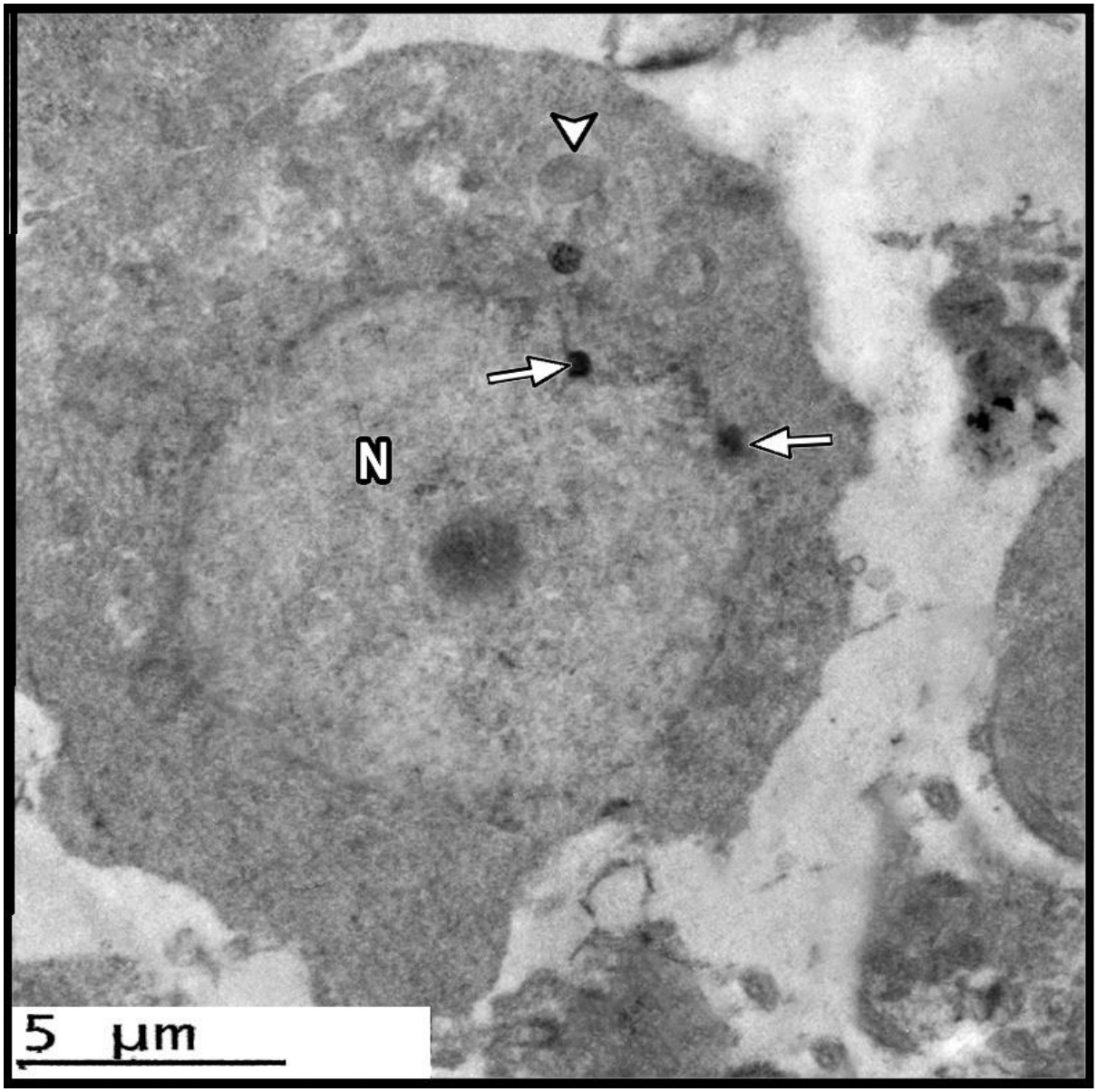
(Fig. 8): Flow cytometric results showing the percentage of expression of Notch1 protein in testicular tissue and cultured spermatogonia. * significant at P < 0.05.



(Fig. 9): A photomicrograph showing: Notch1 positive Aund spermatogonia **(arrows)** and Adiff spermatogonia **(arrow heads). (A) X1000, (B) X1000 and (C) control X1000.**



(Fig. 10): *A photomicrograph showing: Notch1 positive type A spermatogonia* ***(arrows)****.* ***(A) X1000*** *and* ***(B)*** *control* ***X1000****.*



(Fig. 11): Electron micrograph of a testicular tissue showing: Isolated type A spermatogonia with a nucleus **(N)**; Nauges **(arrows)**, mitochondria **(arrowhead).**

Annex 2: Tables.

Gene	Database	Forward primer	Reverse primer
CD44	ENSONIT00000018373.1	GCACCTCACAATGAATGTCCG	ATGGTGCTCTTCTGTTGCTCA
notch1	XM_003451460	CACAAGGCACTGCAGGTTTC	ATTGCTCCTGTGTAACCGGG
gfra1	XM_003441935	AACTGTCGATGCCGAAGAGG	GAGCCGGCTATTCACTGGTT
gapd	JN381952	GGCCATCAATGACCCCTTCA	GCAGACACTTCACCACGGTA

(Table 1): Sequences of primers pairs (5`-3`) used for RT-PCR analysis

Annex 3: References.

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