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Dynamics of Cytokine-like Activity in the Hyperplasic Ovary of Ex-fissiparous Planarians

ABDEL HALIM HARRATH[1,2,*], ABDELHABIB SEMLALI[3], LAMJED MANSOUR[1], WALEED ALDAHMASH[1], SULIMAN Y. AL OMAR[1], MOHAMED S. AL ANAZI[3], JENS R. NYENGAARD[4], AND SALEH ALWASEL[1]

[1] *Department of Zoology, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia;* [2] *Faculté des Sciences de Tunis, UR11ES12 Biologie de la Reproduction et du Développement Animal, Université de Tunis El Manar, Tunis 2092, Tunisia;* [3] *Genome Research Chair, Department of Biochemistry, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia; and* [4] *Stereology and Electron Microscopy Laboratory, Centre for Stochastic Geometry and Advanced Bioimaging, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark*

Abstract. The origin of infertility in the hyperplasic ovary of ex-fissiparous planarians remains poorly understood. In a previous study we demonstrated that a complex process of early autophagy, followed by apoptotic processes, occurs in the hyperplasic ovary of the freshwater planarian *Dugesia arabica*. The present study aimed to investigate whether the mRNA expression levels of selected mRNA-like genes are altered in the hyperplasic ovary of the ex-fissiparous freshwater planarian *D. arabica* compared to the normal ovary. Using human cytokine-specific primers including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), we have successfully amplified by real-time polymerase chain reaction some transcripts that could be similar to those amplified in human. The transcript levels of the human-like transcript (IL-1-like and TNF-α-like) were significantly higher, 4.89- and 3.41-fold, respectively, in the hyperplasic ovary compared to the normal ovary ($P < 0.05$). However, although IL-6-like levels were higher in the hyperplasic ovary than the normal ovary (2.57-fold), this difference was not significant ($P > 0.05$). Immunohistochemical labeling supported the quantitative real-time PCR, showing that, like their respective mRNA expression levels, IL-1, IL-6, and TNF-α-like proteins are more highly expressed in the hyperplasic ovary than in the normal ovary.

Introduction

Fissiparity is a frequent and interesting mode of asexual reproduction that is widespread in freshwater planarians that have no sexual apparatus; hence the term, fissiparous. The acquisition of fissiparity in some species is not necessarily linked to the number or arrangement of chromosomes, as in the case of the model species *Schmidtea mediterranea*, which has 4 chromosomes and incurs translocations from the first to the third pair (De Vries, 1984; Ribas, 1990; Baguna *et al.*, 1999). These translocations are identical and lead to frequent mitotic abnormalities and unstable meiosis. Yet in other species fissiparity seems to be strongly linked to chromosomal mutations (De Vries, 1984; Ribas, 1990), polyploidy (Benazzi, 1974), or aneuploidy (Lepori and Pala, 1982). Some investigations have linked asexuality to the inversion of some genetic factors that otherwise inhibit the transformation of neoblasts into germ cells in fissiparous specimens (Grasso and Benazzi, 1973).

In some cases, fissiparous planarians become sexual after developing a copulatory apparatus, leading to the term, "ex-fissiparous" (Grasso and Benazzi, 1973). Acquisition of sexual maturity by ex-fissiparous specimens is a more or less frequent phenomenon, and the mechanisms have yet to be confirmed. Some studies have revealed the importance of regulators required for germ cell differentiation and maintenance (Salvetti *et al.*, 2005; Wang *et al.*, 2007; Solana *et al.*, 2009). Other studies have identified several prohormones, such as *npy-8*, which is distributed differently in sexual and asexual specimens (Collins *et al.*, 2010). Some ex-fissiparous planarians are completely sterile, because they usually produce infertile cocoons (Stocchino *et al.*, 2012; Harrath *et al.*, 2013). The ovaries become very large, spreading over a wide region of the anterior part of the animal, and are considered to be hyperplasic (Benazzi, 1974; Harrath *et al.*, 2013). Histological investigations have suggested that the remarkable size of these

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* To whom correspondence should be addressed. E-mail: hharrath@ksu.edu.sa

hyperplasic ovaries *versus* normal ovaries is due to a greater proliferation of neoblasts transforming into oogonia (Gremigni and Banchetti, 1972; Benazzi, 1974).

To our knowledge, only two studies have tried to demonstrate the eventual pathological aspects that cause infertility in ex-fissiparous individuals (Gremigni and Banchetti, 1972; Harrath *et al.*, 2014). Consequently, the origin of infertility in the hyperplasic ovary of ex-fissiparous planarians remains poorly understood. In our previous work, our research group demonstrated that infertility in ex-fissiparous planarians may be because oocytes that differentiate in the hyperplasic ovary do not reach maturity, resulting in their being destroyed through programmed cell death (Harrath *et al.*, 2014). Alternatively, our group suggested that cytokine-like molecules may contribute to the occurrence of infertility in ex-fissiparous species. In fact, cytokines are responsible for mediating a large number of physiological processes in metazoan models and mammals, including cell death, growth and differentiation, and developmental patterning (Massagué *et al.*, 2000). Cytokines are important for reproduction, and their expression by oocytes indicates that they have a physiological role in ovarian function, including oocyte maturation and ovulation (Gaafar *et al.*, 2014). Therefore, the deletion of certain genes encoding cytokines could have deleterious effects on male and female reproduction (Ingman and Jones, 2008; Sirotkin, 2011). Using the model organism *Schmidtea mediterranea*, Peiris *et al.* (2014) suggested conservation of the triclad immune system, which may protect against infections, participate during regeneration, and promote wound repair. By homology to macrophages in mammals, planarians have mesenchymal cells, termed phagocytes or reticular cells (Morita, 1991), that may represent a primitive form of macrophage (Peiris *et al.*, 2014). Furthermore, the maresins are a group of molecules that are secreted by mammalian macrophages. This group is important in phagocytosis and has shown up in assays on planarians during regeneration. Exposing planarians to this molecule on injury increases the rate of tissue regeneration (Serhan *et al.*, 2012). This finding suggests that maresins and their signal cascades are conserved between human and planarians (Peiris *et al.*, 2014).

Overall, these data indicate that conservation exists between human and planarian cytokine-like molecules that could be secreted by reticular cells. These data also suggest a link between cytokine-like activity and infertility in planarians. For that reason, dugesiid freshwater planarians provide an excellent opportunity for investigating this rare phenomenon, in particular *Dugesia arabica* Harrath & Sluys, 2013 as a species that exists as both sexual and asexual individuals. In this study we used primers designed for some human cytokines and antibodies against these proteins to detect eventual implications of some cytokine-like molecules in the occurrence of infertility in the ex-fissiparous planarian *D. arabica*. We evaluated the expression levels of IL-1, IL-6, and TNF-α-like molecules in the hyperplasic ovary as compared to normal ovaries. To our knowledge, our study is the first to demonstrate the eventual involvement of cytokine-like molecules in invertebrate reproduction.

Materials and Methods

Light microscopy

Sexual and asexual specimens of *Dugesia arabica* were collected from Yemen, as described previously (Harrath *et al.*, 2013). The hyperplasic ovaries are visible through the dorsal body wall. Thus, we tried to cut the anterior fragments, including the two ovaries, with minimum removal of tissues so as to avoid damaging the ovaries. Anterior fragments of the same size were cut from both ex-fissiparous species and sexual species with normal ovaries. All fragments were fixed in neutral-buffered formalin (NBF) or Bouin's fluid, and were subsequently preserved in 70% alcohol. The sections were cut to a thickness of 7 μm, and were stained in Mallory-Cason.

Total RNA isolation and cDNA synthesis

Transcript levels were studied in freshly prepared mRNA from total fresh planarian tissue of the anterior fragments of planarians, including the ovary. RNA was extracted using the DNA/RNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentrations, purity, and quality were all determined, using the Agilent 2100 Bio analyzer system and Agilent Small RNA analysis kit (Agilent Technologies, Waldbronn, Germany), according to the manufacturer's instructions. The reverse transcription step was conducted on 1 μg of RNA. Using a high-capacity cDNA reverse transcription kit (Applied Biosystems Corp., Foster City, CA), each sample was reverse transcribed into cDNA. The thermal cycling conditions for PCR analysis were as follows: 10 min at 25 °C, 2 h at 37 °C, and 5 min at 85 °C. Then the synthesized cDNA samples were immediately stored at −20 °C.

Quantitative real-time PCR

All experiments on mRNA expression of selected genes were performed in 96-well plates using the CFX96 Real-Time qPCR System (Bio-Rad, Laboratories, Hercules, CA). Expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to normalize the RNA samples to those of the endogenous control. The reactions were carried out, using a PCR SYBR Green Supermix (Applied Biosystems Corp.). Real-time PCR was determined, using a 7500 Real-Time PCR System (Applied Biosystems Corp.). The IL-1 primers were: forward 5′-CTGTCCTGCGTGTTGAAAGA-3′ and reverse 5′-TTGGGTAATTTTTGGGATCTACA-3′. The IL-6 primers were forward 5′-TCTCCACAAGCGCCTTCG-3′ and reverse 5′-CTCAGGGCTGAGATGCCG-3′. The tumor necrosis factor (TNF) primers were: forward 5′-CAGCCTCTTCTCCTTCCTGAT-3′ and reverse 5′-GCCAGAGGGCTGATTAGAGA-3′. The GAPDH primers were: forward 5′-GGTATCGTCGAAGGACTCATGAC-3′ and re-

verse 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′. Primers were added to the reaction mix at a final concentration of 250 nmol l^{-1}. From each sample, 5 μl of cDNA was added to a 20-μl volume of the PCR mixture (0.5-μl-specific primers, 12.5 μl of SYBR Green Supermix (Bio-Rad Laboratories), and 7 μl of RNase-/DNase-free water). PCR cycles were as follows: 50 °C for 2 min and 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 62 °C for 30 min, and 30 s at 72 °C. The results are representative of at least three independent experiments of real-time PCR assays. A single melting temperature peak was used to validate the specificity of each primer pair. The housekeeping gene, GAPDH, was used as a reference gene for this study because it produces uniform expression levels, with less than 0.5-CT (cycle threshold) variation between sample conditions. To confirm that no spurious products were amplified, the amplified products were separated on agarose gel. The obtained results were analyzed using Livak's relative expression method $2^{-\Delta\Delta C_T}$.

Immunohistochemistry

Immunohistochemistry was completed as described previously (Harrath *et al.*, 2014). In brief, fragments of tissues containing ovaries from adult specimens were fixed in NBF fixative, then embedded in paraffin. The sections were made using a classical microtome at 5-μm thickness, and then transferred to coated slides and incubated at 37 °C overnight. Next, the slides were processed with Ventana Benchmark XT (Ventana Medical Systems, Inc., Oro Valley, AZ) and stained with antibodies against human IL-1, IL-2, and TNF-α (Santa Cruz Biotechnology, Dallas, TX), each of which was diluted to 1 : 100. The slides were incubated with the secondary antibody for 1 h at 37 °C, and viewed using the *ultra*View Multimer Detection System (Ventana Medical Systems, Inc.). The slides were then stained with hematoxylin II, then counterstained and mounted with DPX mounting media (Thermo Fisher Scientific, Waltham, MA). Immunostaining of the normal and hyperplasic ovary was quantified using ImageJ software (Schneider *et al.*, 2012).

Statistics

We performed statistical analysis using SPSS ver. 16.0 software for Windows (Statistical Package for the Social Sciences, Armonk, NY) from at least three independent experiments. Bonferroni multiple comparison tests using Stata/SE ver. 13.0.168 (StataCorp, College Station, TX) were also applied. Significance was set as $P < 0.05$.

Results

Light microscopy

While the sagittal section of a normal ovary showed the different stages of oogenesis from oogonia until mature oocytes (Fig. 1A), a section of the hyperplasic ovary showed masses of ovarian cells that were mainly composed of oogonia and young oocytes (Fig. 1B). Because of the intense proliferation of oogonia, hyperplasic ovaries filled almost all of the anterior part of the animal between the intestinal branches (Fig. 1B), which explains the two hyperplasic ovaries in ex-fissiparous *Dugesia arabica* that were much larger than normal ovaries (Fig. 1A, B).

Gene expression levels

In silico *analysis of primer specificity*. Due to the lack of genomic information about *Dugesia arabica*, we tried to check the specificity of the human cytokines specific primers *in silico* using drafts of sequenced planarian genomes and of related worm species. BLAST analysis, throughout the open-access WormBase ParaSite website (Howe *et al.*, 2017), shows specific cross-reactivity of the human cytokine primers with sequences of *Schmidtea mediterranea* coding for proteins with unknown functions. Thus, primers for human IL-1 could amplify the *S. mediterranea'*s sequence having the WormBase scaffold reference "SmedGD_c1.3:v31.007763:32226:32841:-1." Primers of the human IL-6 could amplify *S. mediterranea'*s sequence, having the WormBase scaffold reference "SmedGD_c1.3:v31.003186:7518:8132:-1."

Data are shown as the fold difference in transcript levels measured in the fragments, including comparison of a hyperplasic ovary with a normal ovary. Transcript levels of IL-1-like genes were significantly higher (4.89-fold) in the hyperplasic ovary of ex-fissiparous planarians than in the normal ovary (Fig. 2A, $P = 0.02$). In comparison, the transcript levels of the IL-6-like gene were only 2.57-fold higher in the hyperplasic ovary than in the normal ovary, with this difference not reaching significance (Fig. 2D, $P = 0.08$). TNF-α-like gene transcript levels were significantly higher (3.41-fold) than the normal ovary (Fig. 2G, $P = 0.006$). This result indicates that infertility is associated with increased mRNA levels of cytokine-like proteins.

Immunodetection of cytokines

Figure 2 summarizes the detection of TNF-α, IL-1, and IL-6-like molecules in both hyperplasic and normal ovaries.

IL-1-like protein immunostaining was paranuclear. The intensity of immunostaining was more prominent in the hyperplasic ovary than in the normal ovary, indicating a greater prevalence of oocytes in the hyperplastic ovary (Fig. 2B, C). However, intense staining was detected in the tissue surrounding the normal ovary (Fig. 2B). When comparing IL-6-like protein expressions, expression tended to be higher in oocytes of the hyperplasic ovary (Fig. 2F) than in the normal ovary (Fig. 2E). While immunostaining was most intense in the epithelium and intestine of the normal ovary (Fig. 2H), immunodetection for the TNF-α-like protein was very weak. However, immunostaining for TNF-α-like protein was stronger in

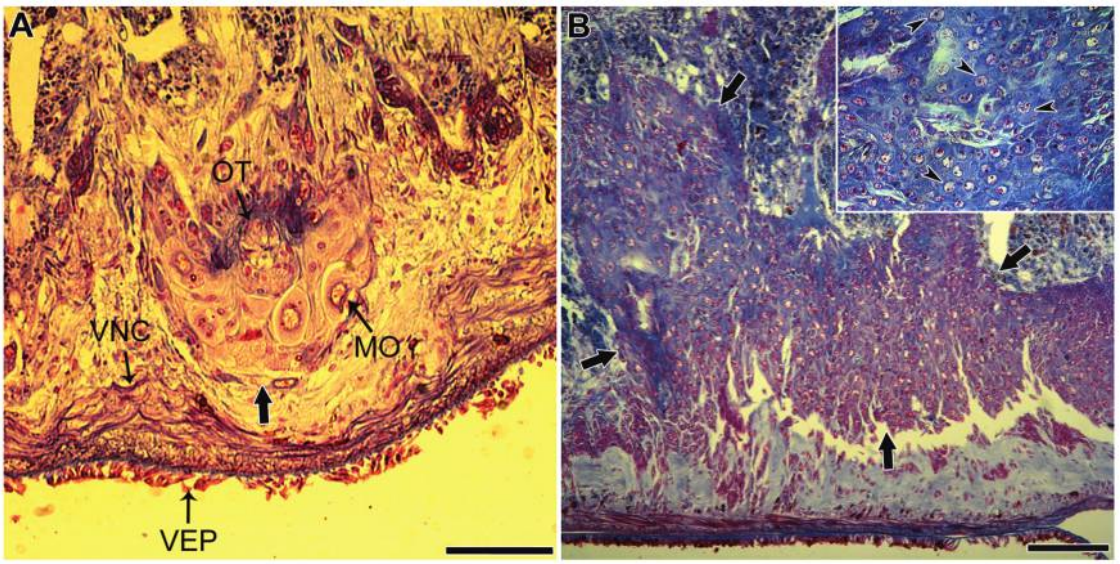

Figure 1. Sagittal section through (**A**) a normal ovary of a sexual *Dugesia arabica* specimen (large arrow) containing oocytes at different stages of maturation, and (**B**) a hyperplasic ovary comprised of masses of cells (large arrows) spread over a wide area of the anterior part of the animal, between the intestinal branches. Insert: magnification of (B) showing the oogonia and young oocytes blocked at the diplotene stage (arrowheads). MO, mature oocyte; OT, oviductal tubule; VEP, ventral epithelium; VNC, ventral nerve cord; scale bar = 200 μm.

oocytes from the hyperplasic ovary than from the normal ovary (Figs. 2H, I).

Discussion

Previously, we hypothesized that the mechanism of cell death that occurs in the hyperplasic ovary of ex-fissiparous *Dugesia arabica* (Harrath *et al.*, 2014) may be linked to the expression of certain proteins. Thus, we set out to improve our understanding of the origin of infertility in the hyperplasic ovary of ex-fissiparous planarians. Specifically, we quantified the expression of some proteins that could play a role in the process of infertility. In preliminary experiments, we successfully tested the amplification of some cDNA using a couple of primers to amplify human cytokines by real-time PCR. Thus, we have formulated the hypothesis that cytokine-like proteins exist in the transcriptome of *D. arabica* and are implicated in the process of cell death in the hyperplasic ovary of ex-fissiparous planarians. BLAST analysis confirmed the annealing of the human cytokine primers with sequences of unknown function in the genome of *Schmidtea mediterranea*. Quantitative real-time PCR using the human cytokine primers shows overexpression of the cytokine-like IL1, IL6, and TNF in the hyperplasic ovary in comparison to the normal ovary. The current study demonstrates that IL-1, IL-6, and TNF mRNA-like gene expression and their proteins are highly elevated in the hyperplasic ovary tissues compared to the normal ovary. Interleukin- and TNF-like molecules have been identified in Annelida, Echinodermata, and Urochordata (Beck and Habicht, 1991). Because cross-reactivity is unlikely to exist between cytokines of different species (Bird *et al.*, 2002), we suggest that these cytokine-like molecules in planarians contribute to the development of infertility in the hyperplasic ovary of ex-fissiparous planarians. This process is realized through induction of apoptosis among growing oocytes (Harrath *et al.*, 2014), similar to that detected in some ovarian disorders of mammals (Figueroa *et al.*, 2015). The immune system clearly contributes to the regulation of gonadal function through the secretion of cytokines, which are closely involved in follicular development and ovulation in mammals (Mori, 1990; Adashi, 1992; Brännström and Norman, 1993). Any dysfunction or overexpression of these cytokines could lead to ovarian disorders.

Cytokines are polypeptide mediators that may be released by immune or nonimmune cells (Beck and Habicht, 1991). It has been suggested—and, in part, demonstrated—that the defense systems of invertebrate hosts are regulated by a network of cytokines analogous to those found in vertebrates (Beck and Habicht, 1991; Huang *et al.*, 2015). In fact, both invertebrates and vertebrates are subject to a variety of environmental insults, such as bacterial infections and wounds that initiate the regulated release of inflammatory cytokines. Thus, the most primitive invertebrates, and probably even protozoa, may have cytokine-like molecules (Beck and Habicht, 1991). Our results showed that transcript levels of IL-1-like genes were significantly higher in the hyperplasic ovary of ex-fissiparous planarians than in the normal ovary. In addition, IL-1-like protein immunostaining was more prominent in the hyperplasic

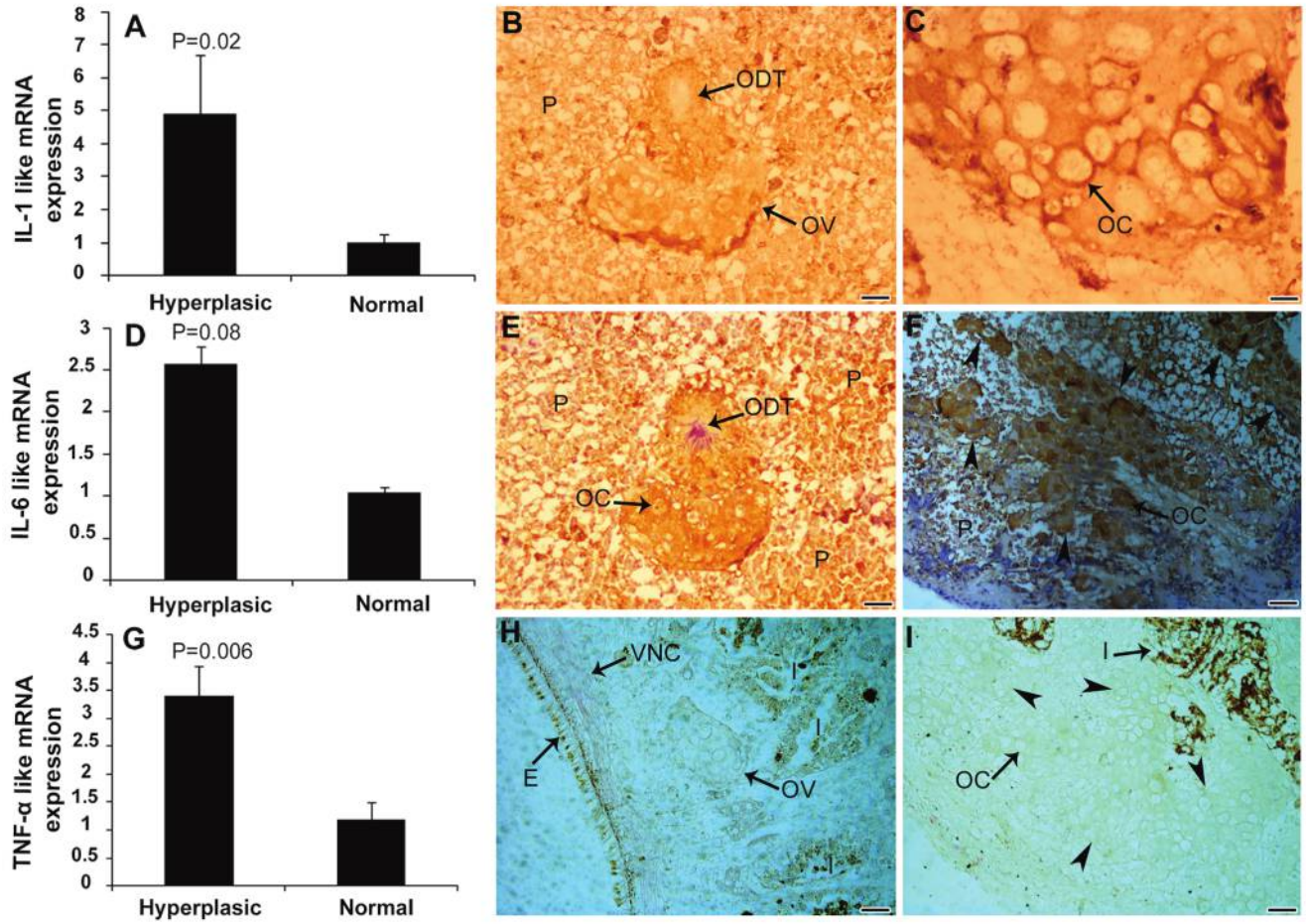

Figure 2. (A) Ovarian IL-1-like mRNA levels in the hyperplasic ovary compared to the normal ovary (P = 0.02). (B)(C) Immunostaining of IL-1-like protein in the hyperplasic ovary (B) compared to the normal ovary (C). (D) Ovarian IL-6-like mRNA levels in the hyperplasic ovary compared to the normal ovary (P = 0.08). (E)(F) Immunostaining of IL-6-like protein in the normal ovary (E) compared to the hyperplasic ovary (F). (G) Ovarian TNF-α-like mRNA levels in the hyperplasic ovary compared to the normal ovary (P = 0.006). (H)(I) Immunostaining of TNF-α like protein in the hyperplasic ovary (H) compaed to the normal ovary (I). Graphical results are shown as means ± standard error of the mean; groups with different letters are significantly different ($P < 0.05$). Scale bars = 100 μm. E, epithelium; I, intestine; OC, oocyte; ODT, oviductal tube; OV, ovary; P, parenchyma; VNC, ventral nerve cord.

ovary than the normal ovary, indicating a greater prevalence of oocytes in the hyperplastic ovary. Isolation of IL-1 molecule from invertebrates, and its assay on vertebrates, has demonstrated that this molecule shares many biological activities with vertebrates, particularly with respect to protein synthesis, prostaglandin, and fibroblast proliferation (Beck and Habicht, 1991). Moreover, IL-1 stimulates phagocytosis and the proliferation of coelomocytes in invertebrate systems (Beck and Habicht, 1991). These processes represent primary host defense cells, also known as hemocytes, amoebocytes, and plasmatocytes (Beck *et al.*, 1989). One study in the last 10 years demonstrated the presence of an innate response in demosponges, which contain key molecules such as toll-like receptors (TLR) and an IL-1 receptor-associated kinase-4-like protein (Wiens *et al.*, 2007). The role of IL-1 in the process of reproduction is still poorly known, even in mammal species (Sirotkin, 2011). Previous studies demonstrated that IL-1 system components have several sites of synthesis in the ovary, such as the oocyte, theca, and granulosa cells (Sirotkin, 2011). It inhibits steroidogenesis in undifferentiated follicles, but stimulates progesterone release in differentiated ovaries (Bornstein *et al.*, 2004). Furthermore, IL-1 may be involved in the synthesis of proteases and the production of nitric oxide and prostaglandin during ovulation. By homology, we believe that the upregulation of IL-1-like molecules in the hyperplasic ovary may induce the proliferation of neoblasts and their differentiation in oogonia, but may inhibit their maturation, causing the pathology of infertility. Other reports

have shown that high levels of IL-33, which is a member of the IL-1 cytokine family and is expressed in many cells and organs (Schmitz *et al.*, 2005), are detected in the serum of patients developing ovarian cancers (Liu *et al.*, 2014; Tong *et al.*, 2016).

In our experiment, TNF-α-like gene transcript levels were significantly higher than the normal ovary, and immunostaining for TNF-α-like protein was stronger in oocytes from the hyperplasic ovary than oocytes from the normal ovary. Tumor necrosis factor expression has been described in invertebrates and has activities resembling those of vertebrates. For example, a gene coding for a TNF-receptor-associated factor has been identified in nematodes (Wajant *et al.*, 1998); and the L929 cytotoxicity assay, which is routinely used to detect vertebrate TNF-α, has also identified TNF in invertebrates (Beck and Habicht, 1991). Tumor necrosis factor tends to be released by phagocytes, and acts on various aspects of the inflammatory response. In particular, TNF is important in necrosis and apoptosis, differentiation, cell proliferation, induction of other cytokines (Rahman and McFadden, 2006), and even in the neoplasia process, which is a major disease affecting cultured flat oysters (Martin-Gomez *et al.*, 2014). For instance, hyperandrogenemia is a primary symptom of polycystic ovary syndrome, characterized by the accumulation of androgens in the ovary without being converted to estrogens (Gonzalez *et al.*, 2012). Due to high serum TNF-α concentrations, this condition can arrest follicular development *via* apoptosis in the granulosa cells, resulting in follicular atresia (Sasson *et al.*, 2002; Jonard and Dewailly, 2004; Escobar-Morreale *et al.*, 2011; Murri *et al.*, 2013; Figueroa *et al.*, 2015; Rezvanfar *et al.*, 2016). Furthermore, women with polycystic ovary syndrome have been described as having higher concentrations of TNF-α in the serum and follicular fluid than normal women (Amato *et al.*, 2003).

In summary, upregulation of the expression of genes encoding cytokine-like molecules (specifically, IL-1 and TNF-α and their relative like-proteins) in the hyperplasic ovary of ex-fissiparous freshwater planarians may indicate the origin of this pathology. Thus, future determination of the complete amino acid sequences of the three studied genes, and their phylogenetic relationships in planarians, is expected to provide interesting evolutionary perspectives.

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