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Original article

Imatinib mesylate does not counteract ovarian tissue fibrosis in postnatal rat ovary

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ABSTRACT

Chemotherapy may result in ovarian atrophy, a depletion of the primordial follicle pool, diminished ovarian weight, cortical and stromal fibrosis. Imatinib mesylate is an anticancer agent that inhibits competitively several receptor tyrosine kinases (RTKs). RTKs play important roles in cell metabolism, proliferation, and apoptosis. In clinic, imatinib mesylate is also known as an anti-fibrotic medicine. In the present study, the impact of imatinib on the ovarian tissue was investigated by assessing ovarian tissue fibrosis in postnatal rat administered with or without imatinib for three days. Fibrosis in the ovarian tissue was determined by histology (Picrosirius and Masson's trichrome staining) and the protein expression of vimentin and alpha-smooth muscle actin (α-SMA). Furthermore, mRNA expression of Forkhead box transcription factor O1 and O3 (FOXO1 and FOXO3), which are markers of cell proliferation was quantified. A short-term exposure to imatinib showed to increase tissue fibrosis in ovaries. This was observed by Masson's trichrome staining. Exposure to imatinib led also to a down-regulation of vimentin protein expression and up-regulation mRNA expression of FOXO3. This may indicate a role of FOXO3 in ovarian tissue fibrosis in postnatal rat ovaries.

1. Introduction

The ovarian cortex is composed by connective tissue cells and collagen fibers, which are surrounding the ovarian follicles of different developmental stages. The medulla is described as highly vascularized stroma usually in the center of the ovary, consisting of connective tissue, muscle cells, elastic and reticular fibers [1,2]. Development and maturation of ovarian follicles are related to a communication between cortex and medulla. Ovarian injury caused by different factors, such as surgery, chemotherapy, radiotherapy, inflammation, and immune abnormalities can trigger ovarian fibrosis [3–5]. Ovarian tissue fibrosis can lead to functional decline of the ovary and even premature ovarian failure [3,5].

Fibrosis is the formation of excess fibrous connective tissue in an organ or tissue, which attributes to the excessive deposition of extracellular matrix components [6,7]. Different factors have been identified as important regulators of fibrosis such as; cytokines (IL-13, IL-21, TGF-β1), chemokines (MCP-1, MIP-1β), angiogenic factors (VEGF), growth factors (PDGF), peroxisome proliferator-activated receptors (PPARs), acute phase proteins (SAP), caspases, components of the renin–angiotensin–aldosterone system (ANG II), and Forkhead box O transcription factors (FOXOs) [5,8–11].

It is shown that some anticancer drugs are able to counteract the cancer cells without causing fibrosis [12,13]. Imatinib mesylate (STI517, Glivec®), a tyrosine kinase inhibitor, is known as a targeted anticancer agent to treat several cancers by blocking receptor tyrosine kinases to inhibit cell proliferation [14,15]. In clinic, imatinib mesylate is also used as an anti-fibrotic medicine, for example during treatment of idiopathic pulmonary fibrosis [13].

The effect of chemotherapeutic agents on ovarian function should include evaluation of the whole ovary including ovarian follicles, cortical tissue and stromal tissue. In our previous study, we have reported

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that imatinib negatively affects early folliculogenesis in postnatal rat ovaries [16]. However, effects of imatinib on ovarian fibrosis on postnatal rats are not known.

In the current study effect of the tyrosine kinase inhibitor, imatinib on ovarian fibrosis were evaluated after short-term in vivo exposure of healthy postnatal rats.

2. Materials and methods

2.1. Animals and study design

All experiments were performed on postnatal Sprague Dawley female rats. The animals (n = 28) were born and maintained under the standard life conditions for 5 days at the Animal Center of the Norwegian Institute of Public Health, Oslo, Norway. Ethical permission for drug treatment was obtained from the Norwegian Institute of Public Health, Oslo, Norway. From day 2 to 4, the treatment groups (n = 20) were treated daily by imatinib mesylate (Glivec ®, STI571, Novartis Pharmaceuticals Corporation, Switzerland) (150 mg/kg) dissolved in water with respect to the rats' weight (1.2–2.1 mg). Both treated and control animals (n = 8) were euthanized on day 5. Bilateral ovariectomy was performed and ovarian samples were collected for histological, immunofluorescence and mRNA expression analysis at the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. The full procedure is described in previous studies [16,17].

2.2. Tissue fibrosis

2.2.1. Picrosirius red staining

To investigate the structure of ovarian tissue, relative areas of fibrosis were evaluated by Picrosirius red staining plus polarization microscopy in Bouins' fixed samples [18]. For this, ovaries from 28 rats (eight from the control group and 20 from the imatinib treated group) were stained. Picrosirius red is a strong anionic dye that attaches to collagen fibers, thus fibrotic areas become easily recognizable [18]. When evaluated by light microscopy, collagen is stained red, while cytoplasm is stained in yellow. Under polarized light microscopy, collagen type I is stained yellow orange birefringence, while collagen type III is stained green birefringence. In brief, collagen types I and III, were calculated as a percentage of the area of each image (expressed in pixels). To avoid counting non-collagen fibers, a color separation on the original (circularly polarized) images, resolving each into its cyan, yellow, magenta and black components. Fibrotic area calculation was performed automatically with the help of the image processing and analysis software Image *J* (National Institutes of Health, USA).

2.2.2. Masson's trichrome staining

Masson's Trichrome stain was performed to determine fibrotic areas in the ovarian tissue by identifying the relative levels of newly formed collagen [18,19]. Ovaries (six from each group, i.c. control and imatinib treated) were fixed in Bouins' solution and embedded in paraffin. The 4 µm deparaffinized sections were first stained with an acid dye (acid fuchsin), treated with phosphomolybdic acid and finally stained with methyl blue. Old collagen was stained in red, while newly formed collagen fibers were stained in blue. Newly formed collagen fibers were stained in blue. Photomicrographs from the Masson's trichrome–stained sections were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera (magnification of 100x). As for Picrosirius red staining, fibrotic area calculation was performed automatically with the help of the image processing and analysis software Image *J* (National Institutes of Health, USA).

2.3. Immunohistochemical and immunofluorescence labelling

The presence of fibrosis in ovarian tissue can be investigated by the determination of protein expression of the structural protein vimentin, as well as alpha-smooth muscle actin (α-SMA) as mesenchymal and myofibroblast markers [18,20,21]. Ovaries (six from each group, i.c. control and imatinib treated) fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned in 4 µm and deparaffinized. The endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck) in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to Phosphate Buffer Saline (PBS). For antigen retrieval, the slides were boiled for 10 min in 10 mM citrate buffer (pH 6.0) in a microwave. The slides were cooled down to room temperature, rinsed with PBS and blocked with 5% goat serum for 30 min at room temperature. The tissue sections were incubated overnight (4 °C) with the primary antibodies: α-smooth muscle actin (α-SMA, Abcam AB5694, 1:100) or vimentin (Abcam AB24525, 1:200) diluted in PBS containing 1% Bovine Serum Albumin. The negative controls lacking the primary antibodies were included. Sections were rinsed with PBS followed by incubation with Alexa-Fluor conjugated secondary antibodies (1:200, Invitrogen). After washing, the sections were mounted with Prolong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific), immunolocalization was visualized and images were acquired using the Keyence BZ-9000 microscope (magnification of 200x). Labelled area was calculated with the help of the image processing and analysis software Image *J* (National Institutes of Health, USA). The relative expression of these proteins was calculated based on the fold difference in their expression in imatinib treated ovaries relative to the control ones.

2.4. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA from postnatal rat ovaries (six controls and 11 from imatinib treated) was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA) followed by PureLink™ RNA Mini Kit (Anbion®), according to the manufacturer's instructions. The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (NanoDrop®2000, Thermo Fisher Scientific Inc., Waltham, MA, USA). For each sample, the RNA concentrations were adjusted to 30 ng/ml and were used to synthesize cDNA. The reverse transcription was performed with the SuperScript III RT-PCR kit (Invitrogen). The mixture was incubated at 42 °C for 1 h, subsequently at 80 °C for 5 min, and finally stored at -20 °C. The negative control was prepared under the same conditions, but without addition of the nucleic acid. qRT-PCR was performed using a thermocycler (iQ5, Bio-Rad, Hercules, CA, USA). Primers (Table 1) derived from the *Rattus norvegicus* gene were designed using the Pick Primers designing tool from the National Center for Biotechnology Information GenBank (NCBI) or based on literature (reference genes; Table 1). A NCBI basic local alignment search tool (BLAST) ensured the specificity of primer sequences. The primers were tested according to the efficiency by qPCR analysis of a dilution series of pooled cDNA at a temperature gradient (55–65 °C) for primer annealing and subsequent melting curve analysis. The reaction mixture for the qPCR contained 0.5 mg cDNA in sterile water (7.4 ml), 10 ml iQSYBR Green Supermix (Bio Rad Laboratories Inc.), forward (0.8 ml) and reverse (0.8 ml) primers (final concentration of 0.4 pmol/ml for each primer), according to the manufacturer's instructions. Data were analyzed using the efficiency corrected Delta-Delta-Ct method (Pfaffl MW, 2001). The fold-change values of the target genes were normalized using the geometric average of the fold-change values of two reference genes: HPRT and SDHA (Table 1). The reactions for PCR amplification consisted of initial denaturation and polymerase activation for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C, 15 s at 95 °C and 1 min at 60 °C. The method qRT-PCR was performed by using the thermal cycler CFX96 real-time PCR detection system (Bio-Rad) and CFX Manager System Software Version 3.0 (Bio Rad). FOXO1 and FOXO3 were used as the markers for cell proliferation [22,23]. The fold-change values of the genes encoding FOXO1 and FOXO3 were

Table 1
Primers used for the mRNA quantification of reference genes (stably expressed) and genes of interest.

Genes	Accession n°	Primer	Product size (pb)	Source
Reference				
HPRT	NM_012583.2	F – GCTGAAGATTTGGAAAAGGTG R – AATCCAGCAGGTCAGCAAAG	157	Hvid et al., 2011
SDHA	NM_130428.1	F – AGACGTTTGACAGGGGAATG R – TCATCAATCCGCACCTTGTA	160	Hvid et al., 2011
Target				
FOXO1	NM_001191846.2	F – TGGGTGTCAGGCTAGGAGTT R – GGCATCTTTGGACTGCTCCT	92	This manuscript
FOXO3	NM_001106395.1	F – AACAGTACCGTGTTCGGACC R – AGTGTCTGGTTGCCGTAGTG	119	This manuscript

normalized using the geometric average of the fold-change values of the two reference genes: HPRT and SDHA (Table 1). The relative expression of FOXO1 and FOXO3 was calculated based on the fold difference in their expression in imatinib treated ovaries relative to the control ones.

2.5. Statistical analysis

In all cases, the statistical tests were performed by using software GraphPad Prism 6. Relative areas of ovarian tissue fibrosis, alpha-SMA, vimentin, as well as mRNA expression were compared between control and treated groups with the non-parametric Mann-Whitney test. All data are presented as mean values ± SEM, the standard errors being calculated from the variance between samples. P-values ≤ 0.05 indicated statistical significance.

3. Results

3.1. Picrosirius and Masson's trichrome staining

Although Picrosirius red staining showed that the total tissue fibrosis increased from 20.1 ± 5.8% in the control group to 39.2 ± 4.1% in the imatinib treated group, this difference was not significant ($P > 0.05$). Similarly, the levels of collagen I increased from 0.26 ± 0.15% in the control group to 0.48 ± 0.12% in the imatinib treated group, while the levels of collagen III increased from 0.23 ± 0.14% in the control group to 0.47 ± 0.13% in the imatinib treated group, but none of these differences were significant ($P > 0.05$) (Fig. 1). However, when the ratio of newly formed collagen: total evaluated tissue section was determined based on Masson's trichrome staining, a significant ($P < 0.0152$) increase in newly formed fibers was observed, where it increased from 0.29 ± 0.11% in the control group to 1.67 ± 0.66% in the imatinib treated group (Fig. 1).

3.2. Vimentin and α-SMA protein expression

Imatinib treatment did not affect α-SMA protein expression. However, a significant ($P < 0.041$) decrease (2.1-fold) was observed when vimentin expression was determined in imatinib treated ovarian tissues when compared with control ones (Fig. 2).

3.3. mRNA expression of FOXO1 and FOXO3

The relative mRNA expression levels of FOXO1 and FOXO3 genes are depicted in Fig. 3. FOXO1 mRNA expression was not affected by imatinib treatment. However, mRNA expression of FOXO3 increased (> 5-fold) in imatinib treated ovaries when compared to control.

4. Discussion

Ovarian fibrosis is characterized by excessive proliferation of ovarian fibroblasts and in addition with the deposition of extracellular matrix, being one of the main reasons for ovarian dysfunction [3,5,24]. A complex network is involved in the development and progression of tissue fibrosis. The exact mechanisms involved in ovarian damage by anticancer treatment are still unclear. However, it is known that ovarian damage includes follicular apoptosis as well as cortical fibrosis [3,24].

Imatinib mesylate is known as an anticancer and anti-fibrotic medicine to treat several cancers by blocking the activity of several RTKs [13–15]. It is suggested that the effect of imatinib may be based on its role as an Abelson tyrosine kinase (c-Abl) inhibitor via PI3K/PTEN/Akt signaling pathways [14,15,25]. There is a controversy in literature about the effect of imatinib on ovarian function. The initial reports have shown the protective effect of this drug on chemotherapy-induced follicle loss in mouse ovary, whereas several other studies have documented inconsistent results [25–30]. Recently, Bildik et al, showed molecular evidence for toxicity of imatinib in human ovary [31].

However, anti-fibrotic effect of imatinib on ovarian fibrosis is still unknown. In the present study, three days imatinib treatment during early folliculogenesis increased tissue fibrosis in postnatal rat ovaries. This was concomitantly with up-regulation mRNA expression of FOXO3 together with a down-regulation of vimentin protein expression.

Fibrosis can obliterate the architecture and function of the underlying organ or tissue, and it is attributed to an excess in the deposition of extracellular matrix components, including collagen, in the tissue [6]. Besides the fact that collagen supports cell structure and architecture in tissues, it also may act as a signaling molecule, activating specific receptors at the cell surface [32].

Among the different methods to assess tissue fibrosis in ovarian tissue, Picrosirius red staining and Masson's trichome are the most used [33–35]. Picrosirius red is an anionic dye that can associate with collagen fibers, which are cationic. Picrosirius red is able to bind the total fibers giving a different birefringence to collagen types I and III in tissues with advanced stages of fibrosis [36]. On the other hand, Masson's trichome staining is used to detect newly formed collagen [19,33]. In a previous study with ovarian autografts, it was possible to observe that the rate of tissue fibrosis detected by Pricosirius Red staining was much higher than that determined with Masson's trichome [35]. Furthermore, Gavish et al. used also Masson's trichome staining to detect early fibrosis in ovarian grafts [33]. These studies confirm that Masson's trichome is more indicated for the assessment of recently formed collagen, as observed in our study. However, none of these methods could show significant changes in collagen I and III in postnatal rat ovaries after exposure to imatinib.

Studies have identified collagen as an endogenous ligand for the discoid in domain receptors 1 and 2 (DDR1 and DDR2) [32,37]. Both DDR1 and DDR2 are tyrosine kinase receptors [38]. Briefly, collagen binding to DDR1 induces tyrosine kinase phosphorylation and may participate in mammary gland formation and breast cancer, whereas DDR2 is activated only by fibrillary collagens, which act as its endogenous ligand [32,37,38]. Matsumura et al. have indicated DDR2 as

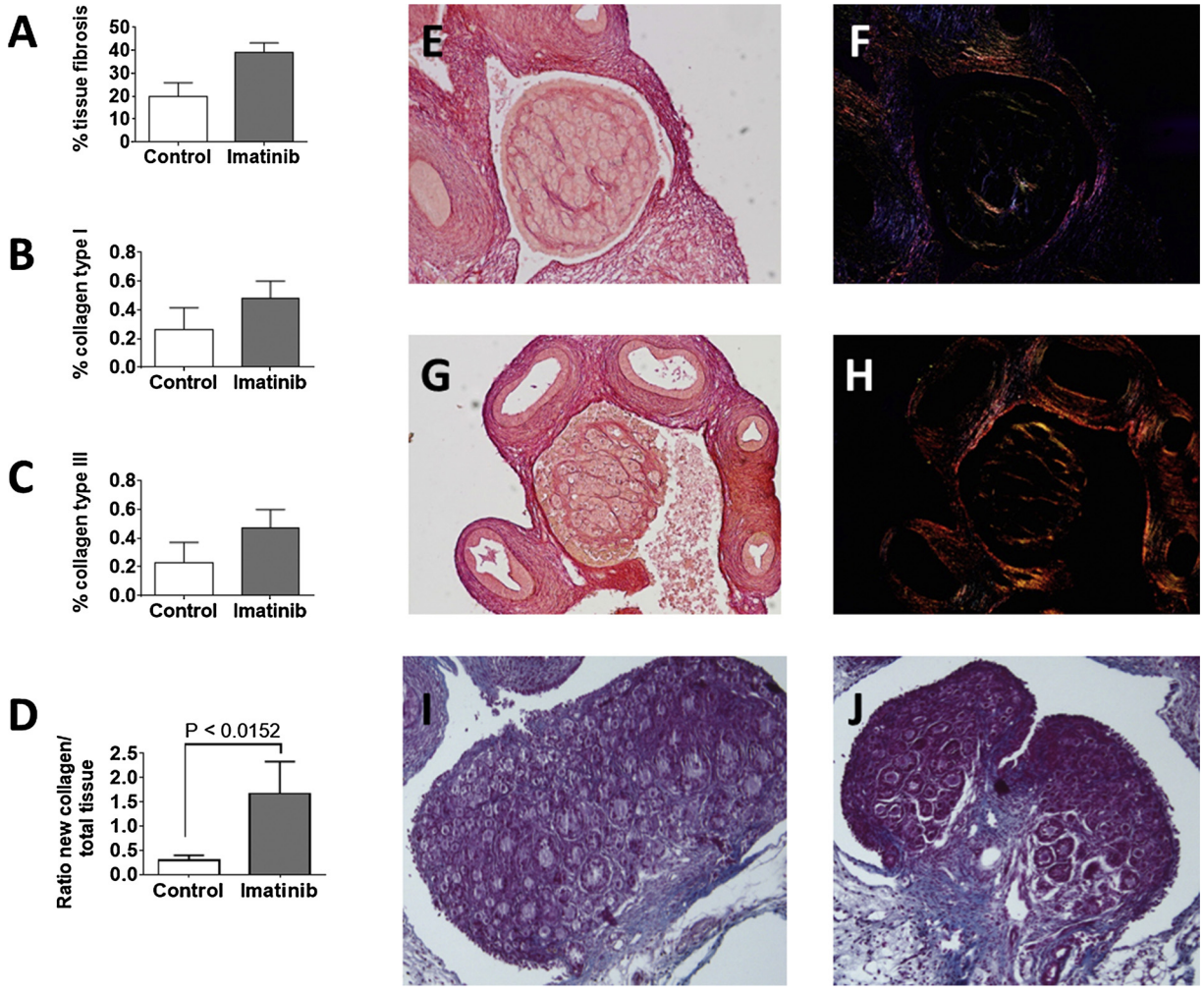

Fig. 1. Total tissue fibrosis, collagen type I and III in postnatal rat ovarian tissue from control and treated with imatinib, based on Picrosirius red staining (A, B, C) and ratio of newly formed collagen and total tissue, based on Masson's trichrome staining (new collagen in blue) (D). Images in panels represent postnatal rat ovarian tissue from control (E, F, I) and treated imewith imatinib (G, H, J).

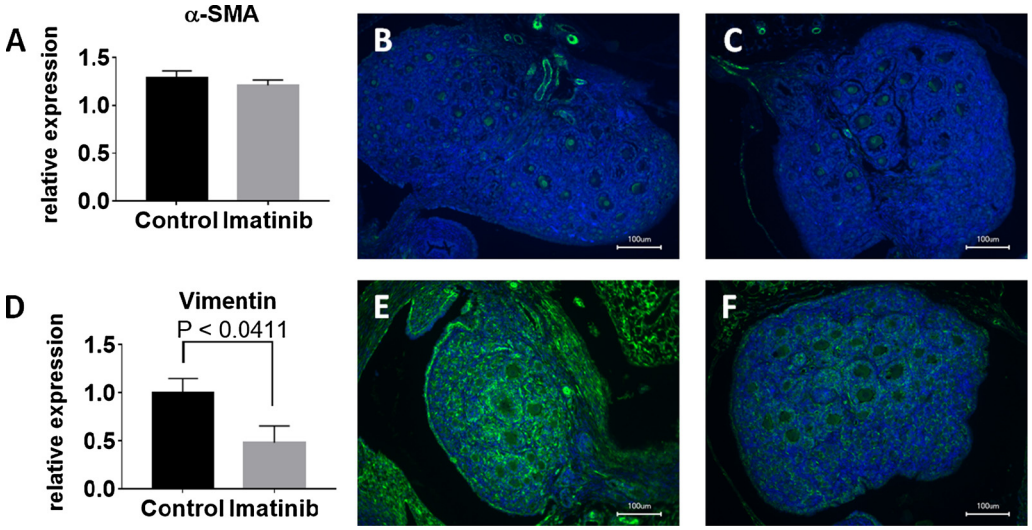

Fig. 2. Immunolabelling of α-SMA (A–C) and vimentin (D-F) proteins in ovarian tissue from control group (B, E) and treated with imatinib (C, F). The relaive expression of these proteins is expressed as fold-change in levels of α-SMA (A) and vimentin (D) in postnatal rat ovaries from control and imatinib treated animals.

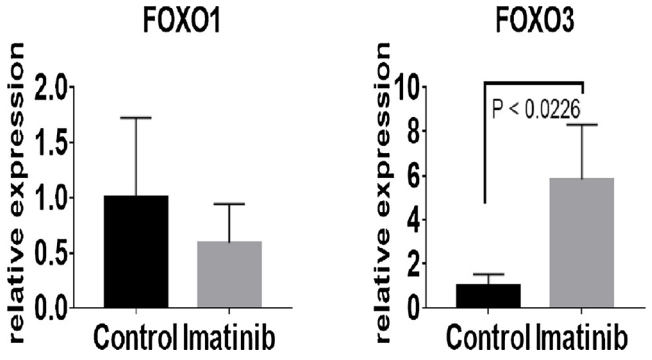

Fig. 3. The fold-change in mRNA expression levels of FOXO1 (A) and FOXO3 (B) genes in postnatal rat ovaries from control and imatinib treated animals.

a critical player in ovarian function and regulation, such as anti-apoptosis, steroid synthesis, gonadal development, ovulatory cycles and ovulation [39]. Imatinib is known to inhibit DDRs and regress fibrosis in mature mammals with cancer [40,41]. In the present study, no significant differences of collagen types I and III between imatinib treated group and the control groups were observed in postnatal rat ovary during three days treatment (Fig. 1). However, differences were observed in the early tissue fibrosis. We may conjecture that the total amount of collagen may increase over time. Further studies with a longer treatment duration may show different results.

FOXO transcription factors are key regulators of cellular metabolism, proliferation, apoptosis and stress resistance [22]. In ovaries, inactivity or mutation of these transcription factors have been related to premature ovarian failure [42]. Transcription factors FOXO1 and FOXO3 in rodents are highly expressed respectively in granulosa cells and oocytes at different stages of follicle development [43]. They play a role, not solely on primordial follicle activation, but also on oocyte maturation, ovulation and luteinization [43,44]. Inactivity or mutation of these transcription factors has been related to POF [42]. The effect of imatinib is based on binding to c-Abl kinase and inactivating the PI3K/Akt/FOXO axis [28,45]. FOXOs have also shown to play a potential role in tissue fibrosis in other organs e.g. heart, lungs and liver [11,23,46,47]. As far as we know, the role of FOXOs in ovarian tissue fibrosis has not been previously described.

In this study, FOXO1 was not affected, while FOXO3 was up-regulated in the imatinib treated postnatal rat ovaries. Activated Akt phosphorylates a variety of effector molecules including FOXO1, which then control cell survival and growth [48]. FOXO1 is mostly known as regulator of cell cycle progression and apoptosis via KL/c-Kit and PI3K signaling pathways [43]. FOXO1 is important for wound healing by decreasing fibroblast proliferation [49,50]. Akt phosphorylates and inactivates FOXO3a by promoting translocation to the cytoplasm, which promotes proliferation [48]. Studies on the role of FOXO3 on fibrosis suggest that inactivation of this factor is associated with a decreased fibrosis, probably by regulating proliferation and apoptosis sensitivity [51–53]. FOXO3a deficiency protects idiopathic Pulmonary Fibrosis fibroblasts from type I polymerized collagen matrix-induced apoptosis *via* caveolin-1 and Fas [11].

Imatinib inhibit FOXO3 phosphorylation, i.e. its translocation to cytoplasm of primordial follicles *in vitro* via PI3K/Akt pathway [45,54]. However, besides phosphorylation, FOXO3 can be also regulated by acetylation, methylation, glycosylation, redox modulation, and ubiquitination, which will determine FOXO3 function [55]. The higher expression of FOXO3 in imatinib treated postnatal rat ovaries may be related to ovarian tissue fibrosis in the current study. This is contradictory with the anti-fibrotic effect of imatinib on other type of tissues compared with ovarian tissue in postnatal rats [51–53,56]. Therefore, the precise role of FOXO3 in ovarian tissues remains unclear, and it could be reasonable to postulate that FOXO3 may have another role in ovarian tissue fibrosis in postnatal rats. In spite of high similarity among RTKs, it seems there are differences within their activation, which could be exploited to obtain kinase selective compounds. These differences may depend on species selection and/or some other factors such as age, type of tissue, dose and duration of treatments.

During fibrogenesis, mutually interacting factors disrupt the balance between synthesis and degradation of extracellular matrix [5]. In addition, over-proliferation of ovarian mesenchymal fibroblasts and the excessive deposition of extracellular matrix may be stimulated [5]. Imatinib inhibits the vascular endothelial growth factor (VEGF), which in its turn plays a role in fibrosis by upregulating the expression of collagen and α-SMA [5,57]. Luo et al. found that α-SMA directly regulates several profibrotic and immune cytokine genes in hepatic stellate cells [58]. Protein α-SMA is associated with enhanced collagen secretion and, therefore, represents a critical transition in fibrogenesis [59]. (Challa and Stefanovic 2011, Hinz 2016) In the present study, imatinib did not affect the expression of α-SMA. This shows that fibrosis may occur via a different pathway. On the other hand, vimentin expression was reduced significantly in imatinib treated postnatal rat ovaries. Vimentin filaments are important in the development of tissue fibrosis by stabilizing the collagen I and promoting fibroblasts migration [60,61]. Disruption of vimentin reduces synthesis of type I collagen [60]. From a therapeutic point of view, the increased collagen expression has a central role in the development of tissue fibrosis [60]. Vimentin filaments are known as a target for anti-fibrotic therapy where imatinib treatment prevents tissue fibrosis by reducing the vimentin expression [60,62]. As expected, the present study showed a decrease in vimentin expression caused by imatinib administration. However, without avoiding ovarian tissue fibrosis. Vimentin is involved in sequestering and degradation of FOXO3 [61]. Hence, this finding shows that fibrosis does not only follow a vimentin-dependent pathway in postnatal rat ovaries and other targets than collagen type I should be considered.

The present study suggests that anti-fibrotic effects of imatinib in ovaries may be age-dependent. Observation is in line with previous reports where imatinib has shown to interfere with physiological signal cascades and this modulation has varied significantly with age and maturational status [17,63]. However, further studies are needed to evaluate differences in mechanism of ovarian fibrosis at different maturational stages with a longer treatment duration. Consequently, before introducing imatinib as an anti-fibrotic drug we need more information about the complicated mechanism action of this drug in different tissues and its possible age-dependent adverse effects on ovarian stroma.

5. Conclusion

In conclusion, increased tissue fibrosis in postnatal rat ovaries treated with imatinib is probably related to a down-regulation of vimentin protein expression and up-regulation mRNA expression of FOXO3. More investigation of other related up/downstream proteins involved in fibrosis in postnatal rat ovaries might strengthen this observation.

Conflicts of interest

The authors declare no conflicts of interest.

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Transparency document

The Transparency document associated with this article can be found in the online version.

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