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Insulin-like growth factor-1 isoforms in human ovary. Preliminary report on the expression of the IGF-1 gene in PCOS patients and healthy controls

Izoformy insulinopodobnego czynnika wzrostu-1 w jajniku ludzkim. Doniesienie wstępne na temat ekspresji genu IGF-1 u kobiet z zespołem policystycznych jajników (PCOS) i zdrowych

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Abstract

Insulin-like growth factor 1 (IGF-1), produced and secreted locally, may affect the mechanisms of folliculogenesis and cause ovarian dysfunction, characteristic of PCOS. The expression of the IGF-1 gene gives rise to three different isoforms of the original molecule. Until now, the role of IGF-1 isoforms has been documented in the repair processes of damaged muscle fibers, cardiomyocytes, hepatocytes, and neurons. The literature offers no reports on the presence and role of IGF-1 isoforms in the ovary.

Objectives: *The aim of the study was to assess the IGF-1A, B and C isoforms at the level of IGF-1 gene transcription in the ovaries of PCOS women and healthy controls.*

Material and methods: *Serum samples and ovarian tissues from PCOS women, treated and non-treated with metformin (PCOS M(+);n=12 and PCOS M(-),n=37, respectively), and controls (n=21) were obtained. The expression of mRNA species of IGF-1 in the ovaries was determined by quantitative RT-PCR.*

Results: *The presence of transcripts of three types of IGF-1 isoforms was observed in healthy controls and PCOS patients, regardless of metformin treatment. Total expression of all isoforms was higher in the M(-)(Me-26640) group as compared to the M(+)(Me-13470) group, as well as controls (Me-17030)-(not significant, p=0.061). Similar results for IGF-1A were obtained in all groups. The relative expression of IGF-1A was lower in the M(-)(86.02%) group and differed statistically from controls (91.38%) (p=0.011).*

Conclusions: *We detected the presence of mRNA for three IGF-1 isoforms in human ovary. To the best of our knowledge, this has been the first report on the presence of mRNA for three IGF-1 isoforms in human ovary. We found differences in the relative expression of IGF-1A isoforms between the investigated groups.*

Key words: **IGF-1** / **isoforms** / **PCOS** / **insulin-like growth factor - 1** / **ovary** / / **alternative splicing** /

Streszczenie

Insulinopodobny czynnik wzrostu 1 (IGF-1) produkowany i wydzielany lokalnie może wywierać wpływ na zaburzenia procesu folikulogenezy oraz powodować dysfunkcję jajnika charakterystyczną dla PCOS. W wyniku ekspresji genu dla IGF-1 powstają trzy izoformy pierwotnego transkryptu – A, B oraz C. Według dotychczasowych doniesień, zmianę ekspresji izoform udokumentowano w procesach naprawczych uszkodzonych włókien mięśniowych, kardiomiocytach, hepatocytach i neuronach. W dostępnych bazach naukowych nie odnotowano informacji na temat obecności, czy roli izoform IGF-1 w jajnikach ludzkich.

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Cel: *Jakościowa i ilościowa ocena izoform IGF-1A, B oraz C na poziomie transkrypcji genu IGF-1 w jajnikach kobiet z zespołem PCOS oraz zdrowych.*

Materiał i metody: *Analizowano skrawki jajników oraz surowice pacjentek z zespołem PCOS nieleczonych metforminą (PCOS M(-), n=37), kobiet z PCOS leczonych metforminą (PCOS M(+); n=12) i zdrowej grupy kontrolnej (n=21). Ekspresję mRNA dla poszczególnych frakcji izoform oceniano za pomocą reakcji odwrotnej transkrypcji RT-PCR.*

Wyniki: *Na podstawie uzyskanych wyników, udowodniono obecność transkryptów genu dla izoform A, B oraz C insulinopodobnego czynnika wzrostu-1 w jajnikach ludzkich. Zsumowany poziom ekspresji wszystkich izoform był najwyższy w grupie M(-)(Me-26640), w porównaniu do M(+)(Me-13470), a także do grupy kontrolnej (Me-17030) -(brak istotności, p=0,061). Podobne wyniki uzyskano analizując tylko poziom ekspresji IGF-1A. Ponadto poziom ekspresji izoformy IGF-1A pod względem udziału procentowego różnił się statystycznie między grupami M(-) (86,02%) i kontrolną (91.38%)(p=0,011).*

Wnioski: *Wykazano obecność transkryptów dla trzech typów izoform IGF-1 w jajnikach ludzkich. Opierając się na dostępnym piśmiennictwie należy stwierdzić, że jest to pierwsza uzyskana w tym zakresie badań obserwacja. Odnotowano różnice w udziale procentowym ekspresji IGF-1A między grupami.*

Słowa kluczowe: **IGF-1** / **izoformy** / **PCOS** / **Insinsulinopodobny czynnik wzrostu – 1** / / **jajnik** / **alternatywny splicing** /

Introduction

Polycystic ovary syndrome (PCOS) is a heterogenous disorder and has been a point of interest for the scientific and medical world for years. It was first described eight decades ago by Stein and Leventhal [1], and consists of a spectrum of metabolic disorders and accompanying clinical symptoms. Nowadays, the search for the 'PCOS' term at the US National Library of Medicine – PubMed generates over 6.8 thousand results [2], indicating the significance of this problem not only for the researchers but also for patients, and the general population of women. According to the 1990 NIH definition, PCOS affects approximately 6-10% [3] of all women at the reproductive age worldwide. However, according to the Rotterdam criteria, this problem appears to be much wider [4]. The ESHRE / ASRM – 2003 consensus [5] definition of PCOS remains a subject of much controversy and does not reflect the actual clinical problem which PCOS, together with the whole spectrum of the associated symptoms, presents. Currently, a significant role in the pathogenesis of PCOS is attributed to insulin resistance and compensatory hyperinsulinemia [6], as key factors in disease emergence, determining the clinical picture as well. However, it seems that the mechanism of the effect of insulin on the pathogenesis of PCOS has been only partially understood. It is probably due to the fact that a large number of dependencies and cross-receptor reactions in which insulin participates significantly impede the interpretation of the problem. Hyperinsulinemia affects 30-80% of PCOS women in all BMI groups [7], but due to inaccuracy of the tests for insulin-sensitivity and insulin-resistance the problem appears to be largely underestimated. Numerous therapies have been implemented to treat PCOS symptoms, including oral contraceptive pills (OCP), metformin, and statins [8]. However, recent research revealed that, apart from metformin, the widely used drugs may even worsen the atherogenic profile (OCP) [9], or alter insulin sensitivity (statins) [10]. The inability to find an effective therapy propels further research on the pathogenesis of PCOS.

The influence of the endocrine and paracrine factors on the ovarian function is undeniable, but hardly explained. Research in this field constitutes a considerable challenge, probably due to the large number of molecules involved in the regulatory processes. However, some disturbances in cytokines and growth factors in PCOS ovaries have been defined. Besides insulin, there is an alteration in the insulin-like growth factors (IGFs), tumor necrosis factor- α (TNF- α), and interleukins 6 and 18 (Il-6, Il-18) [11, 12, 13, 14]. These factors may play a role in mitogenic and proliferative processes in granulosa and theca-interstitial cells or mechanisms of ovarian steroidogenesis directly.

The molecule which is structurally and functionally associated with insulin is the insulin-like growth factor-1 (IGF-1). The abnormal metabolism of this factor in PCOS has been proven. Increased free plasma fraction of IGF-1 plays an important role in the failure of ovarian androgenesis, not only with the synergistic action with LH, but also influencing the process of the programmed cell death and proliferation of T-I compartment in PCOS ovaries [15, 16, 17, 18]. Locally produced and secreted IGF-1 may affect the mechanisms of folliculogenesis, inducing a larger number of follicles to grow. IGF-1 is a peptide hormone with structural similarity to proinsulin, characterized by a wide spectrum of endocrine, autocrine and paracrine action. It is also called 'somatomedin C' and is considered the main regulator of postnatal growth of an organism. Common data indicate that it is mainly synthesized in the liver (approximately 70% of the total circulating pool), but also locally, in all body tissues [19]. Nowadays, a growing amount of research focuses on the role of IGF-1 produced locally, as a highly active form of a peptide regulating cellular processes. Overexpression of the IGF-1 gene and elevated levels of the mature peptide have been observed in pathologies such as endometriosis or fibroids, and in some malignant tumors [20]. The mere expression of the IGF-1 gene is subject to extremely sensitive molecular regulation, including the so-called 'alternative mRNA splicing', which gives rise to three isoforms of the original IGF-1 molecule. Alternative splicing is a regulated process which takes place during gene expression and results in the creation of multiple proteins from a single gene coding. In this process, particular pre-mRNA exons or introns

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may be assembled not only according to the gene pattern, but also included within or excluded from the final, processed messenger RNA (mRNA) produced from that gene [21].

The IGF-1 gene consists of 6 exons, divided by introns – long non-coding sequences. Exons 1 and 2 are the leader sequences dividing transcripts into two classes – 1 and 2. Exons 3 and 4 encode the conservative mature IGF-1 peptide, and post-translationally cut E-peptide is encoded by exons 5, 6 and partially by 4 [22, 23]. (Figure 1).

As mentioned above, alternative splicing of the IGF-1 gene creates two classes of isoforms – 1 and 2 (including A, B and C isoforms in each), out of which only class 1 (IGF-1A, IGF-1B and IGF-1C) seems to be biologically significant. The result of alternative splicing is post-translational variability in the protein structure originating from one gene. The change in the amino acid pattern may influence bioactivity or localization of the molecule in the cells. It is estimated that 35%-90% of human genes are being alternatively spliced [24].

Until now, the role of IGF-1 isoforms has been documented in the repair processes of damaged muscle fibers, cardiomyocytes, hepatocytes, and neurons [25]. To the best of our knowledge, there have been no reports on the occurrence and the role of IGF-1 isoforms in the ovary.

Objectives

The aim of the study was qualitative and quantitative assessment of the IGF-1A, B and C isoforms at the level of transcription of the gene for IGF-1 in the ovaries of women diagnosed with PCOS and healthy controls. The following steps were undertaken:

1. Qualitative analysis of the presence of transcripts of the gene for the IGF-1 protein at the level of mRNA splicing variants in human ovaries.
2. Quantitative analysis of transcripts of the IGF-1 gene-specific isoforms of IGF-1A, IGF-1B, IGF-1C in the ovaries of women:
   a. diagnosed with PCOS
      – non-treated with metformin – PCOS M (-)
      – treated with metformin – PCOS M (+)
   b. healthy controls

Materials and methods

The material consisted of samples of the ovaries and blood obtained from 72 women. The study group included 49 patients at the reproductive age, with PCOS diagnosed according to the ESHRE / ASRM criteria – Sponsored PCOS Consensus Workshop Group, Rotterdam 2003, in accordance with the Fertility and Infertility Section of the Polish Gynecological Society Guidelines. The patients were deemed eligible for an ovarian wedge resection by laparoscopy after a lack of the ovarian response to multiple (3-6 cycles) clomiphene citrate stimulation or treatment with pituitary gonadotropin activity hormones. Since metformin had been used in some patients, the group was retrospectively subdivided into: group 1 – patients diagnosed with PCOS – non-treated with metformin in the 12 months prior to enrollment PCOS M(-) (n=37), and group 2 – patients diagnosed with PCOS – treated with metformin during enrollment, PCOS M(+) (n=12).

The control group (n=21) consisted of patients at the reproductive age:

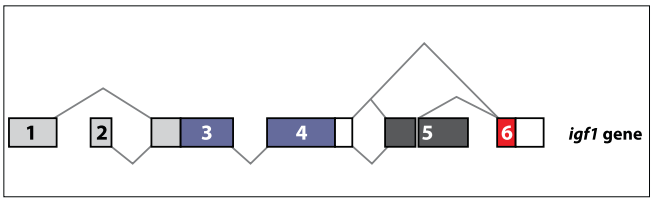

Figure 1. IGF-1 gene structure. Leader sequences 1 and 2; Exons 3 and 4 encoding mature IGF-1 peptide; and exons 5 and 6 encoding the E-peptide.

a) operated on because of a lesion in the ovary identified during a physical examination and vaginal ultrasonography,
b) operated on to broaden the diagnosis of the causes for infertility, in whom lesions were observed during surgery in the ovary.

Both above required of the operator to remove the lesion with a margin of the healthy tissue, perform biopsy of the healthy tissue and/or the healthy ovary.

Controls did not meet any of the diagnostic criteria for the diagnosis of PCOS according to the ESHRE/ASRM criteria – Sponsored PCOS Consensus Workshop Group 2003.

The exclusion criteria for both groups were as follows: ovarian, adrenal, endometrial, cervical and breast neoplasms, pelvic endometriosis, congenital adrenal hyperplasia, clinically and/or laboratory-confirmed endocrine disease (thyroid dysfunction, acromegaly, gigantism, Cushing's disease), diabetes type I or II, hyperprolactinemia, and unexplained vaginal bleeding.

The objectives of the work were carried out by clinical assessment, hormonal and biochemical status, immunohistochemical confirmation of the presence of the mature protein IGF-1 in the ovaries, and the assessment of molecular quantities of transcripts of IGF-1 gene-specific isoforms of IGF-1A, IGF-1B and IGF-1C.

The evaluation of the women included detailed clinical and biochemical profile to diagnose or exclude PCOS and other endocrinopathies:

- analysis of the menstrual cycle by observing the intervals between the periods in the last 12 months,
- evaluation of clinical hirsutism as the manifestation of hyperandrogenization using the modified Ferriman-Gallwey scale,
- ultrasound examination of the ovaries,
- hormonal and biochemical evaluation: serum levels of FSH, LH, PRL, testosterone, SHBG, 17OH-P, E2, and DHEA-S – for further studies.

After signing a written consent, subjects who met the inclusion criteria were enrolled. A small patch of tissue was collected from the sample removed during surgery, frozen in RNAlater RNA Stabilization medium Reagent (Qiagen) in 1.5 ml tubes at -80° C, in order to preserve the largest amounts of RNA in the preparation. The homogenate obtained from the sample was subjected to RNA isolation using RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions, and then studied by biomolecular techniques (RT-PCR) for the presence of three different transcripts of the IGF-1 gene, which are translated into three isoforms of the IGF-1 molecule. The absolute number of copies was obtained for individual isoforms in a given sample. The study was performed at the Department

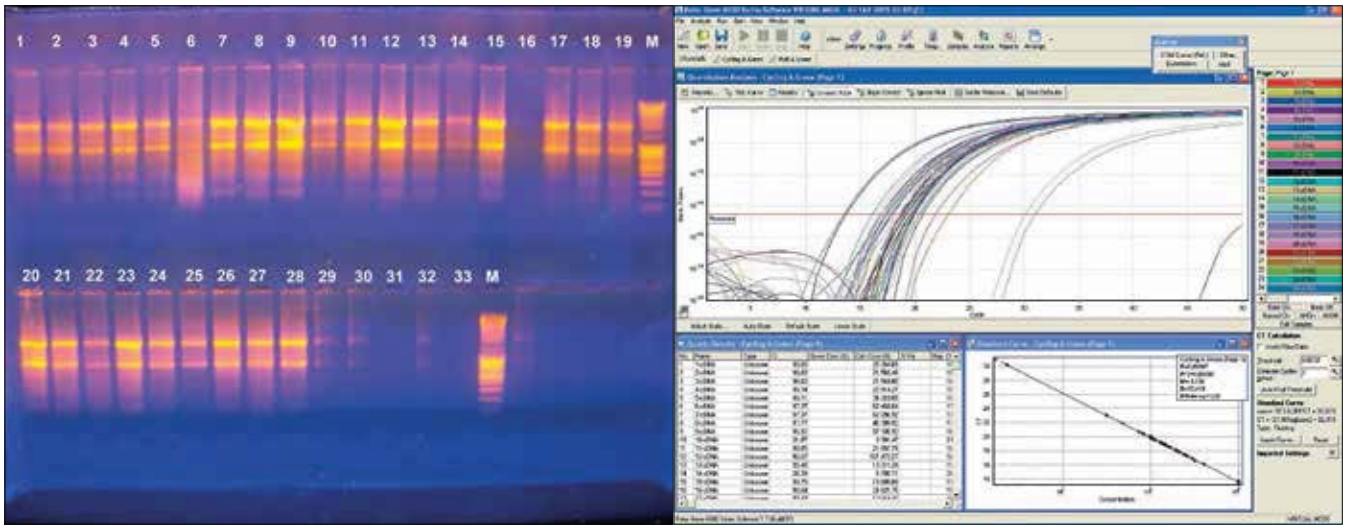

Figure 2. Isolation of RNA –electrophoresis in agarose gel and RT-PCR chart identifying isoforms of IGF-1.

of Molecular Virology, Adam Mickiewicz University. In order to confirm the presence of IGF-1, the procedures also included a qualitative analysis of the mature peptide of IGF-1 in ovarian tissue of patients from the study group and controls, using immunohistochemical techniques at the Department of Pathology, Poznan University of Medical Sciences, at the Obstetrics and Gynecology University Hospital.

Statistical analysis was performed using non-parametric tests U Mann-Whitney, ANOVA Kruskall-Wallis and post-hoc Dunn's test. All calculations were performed using GraphPad Prism, ver. 5.00, GraphPad Software, Inc. Nonparametric data were presented due to lack of normal distribution of the variables.

Results

The presence of transcripts of all types of IGF-1 isoforms (IGF-1A, IGF-1B and IGF-1C) was observed in the analyzed fragments from the ovaries of healthy controls and PCOS women, regardless of metformin treatment. (Figure 2).

As far as the total expression of copies of transcripts for all isoforms was concerned, their number was the highest in PCOS women non-treated with metformin PCOS M(-) (Me-26640), as compared to those treated with metformin PCOS M(+) (Me-13470), and controls (Me-17030), but the differences were statistically insignificant (p=0.061). (Figure 3).

Assessment of the number of copies of transcripts for the A isoform in each group revealed that their number was the highest in ovaries derived from PCOS women non-treated with metformin (Me-23150), although the differences were statistically insignificant as compared to patients treated with metformin (Me-12160), and controls (Me-16150) (p=0.095). (Figure 4).

The examination of the percentage of each fraction (IGF-1A, IGF-1B and IGF-1C) in the total expression of transcripts revealed that in all groups the largest percentage of the total was represented by the IGF-1A isoform (from 86.02% in the PCOS group M(-) to 91.38% in the control group), whereas the smallest percentage was represented by the IGF-1 C isoform (0.81% in the control group to 1.25% in the PCOS group M(-). (Figures 5, 6, and 7).

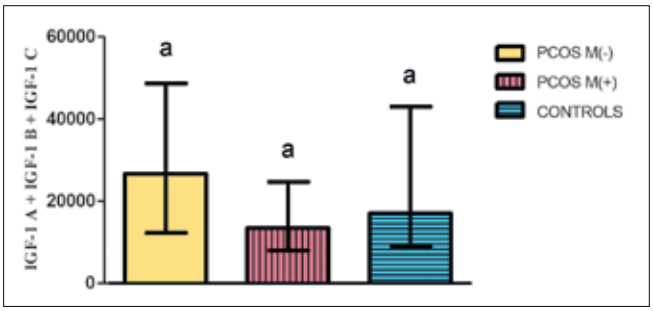

Figure 3. Comparison of the total expression of the copies of transcripts for all isoforms revealed their number was the highest in PCOS women non-treated with metformin as compared to patients treated with metformin and controls. In the analyzed subgroups, lack of common letters between the columns on the chart signifies statistical significance. The level of the column indicates the median, lines – interquartile range.

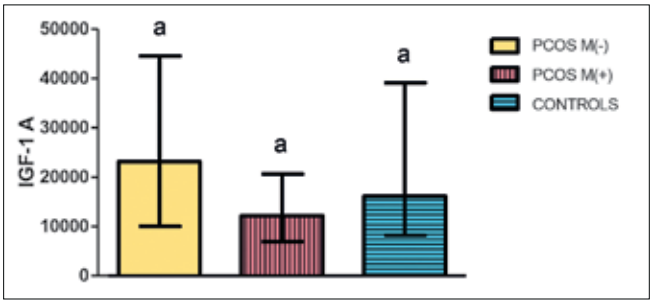

Figure 4. Comparison of the expression of the copies of transcripts for the IGF-1A isoform revealed their number was the highest in PCOS women non-treated with metformin as compared to patients treated with metformin and controls. In the analyzed subgroups, lack of common letters between the columns on the chart signifies statistical significance. The level of the column indicates the median, lines – interquartile range.

A statistically significant difference in the proportion of IGF-1A isoform of the total between the two groups was demonstrated (p=0.011). The proportion of IGF-1A isoforms was found to be the lowest in the PCOS group M(-) (86.02%) and statistically different from the control group (91.38%). No statistical significance was observed between the PCOS group M(+) (90.78%), and the control group, or between PCOS M(-) and PCOS M(+).

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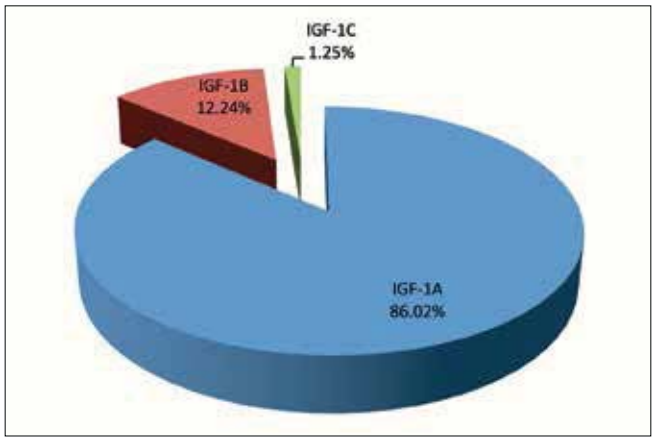

Figure 5. The percentage of each fraction (IGF-1A, IGF-1B and IGF-1C) in the total expression of transcripts of mRNA in the PCOS subgroup non-treated with metformin PCOS M(-).

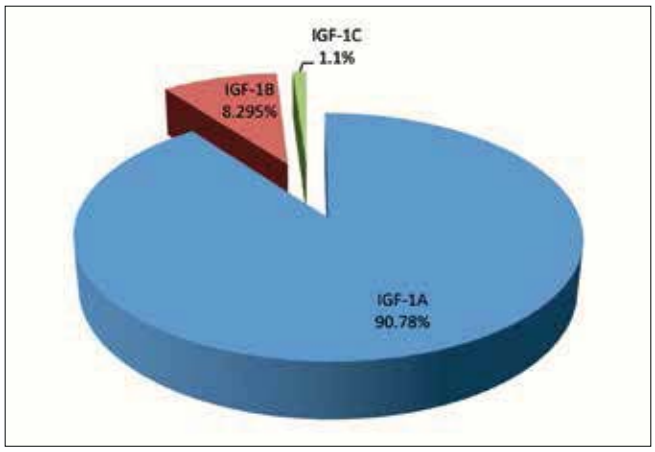

Figure 6. The percentage of each fraction (IGF-1A, IGF-1B and IGF-1C) in the total expression of transcripts of mRNA in the PCOS subgroup treated with metformin PCOS M(+).

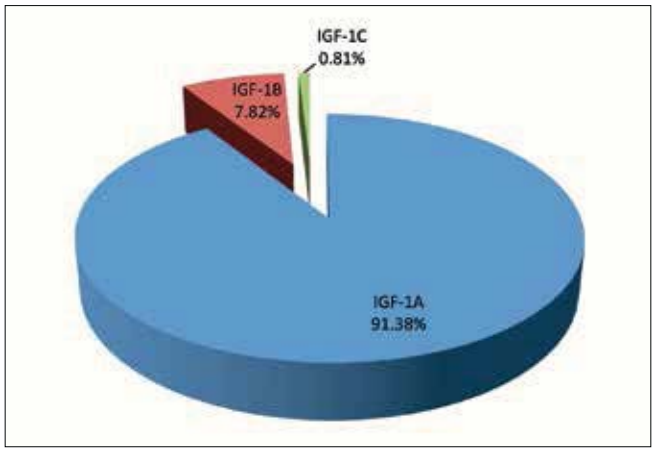

Figure 7. The percentage of each fraction (IGF-1A, IGF-1B and IGF-1C) in the total expression of transcripts of mRNA in the control group.

Table I shows medians, minimum, maximum and interquartile ranges of the expression of IGF-1A isoforms in each group. Nonparametric data were calculated due to lack of normal distribution of the variables.

Table I. The comparison of the proportion of the IGF-1A isoform of the total (%) in individual groups. Values are shown as median, upper and lower quartile and maximum, minimum (p<0.05 Kruskal-Wallis test).

Group / % IGF-1A	PCOS M(-)	PCOS M(+)	Control	P
Minimum	73,99	81,82	74,09	-
25% percentile	83,63	89,16	88,82	-
Median	**86,02***	**90,78**	**91,38***	**<0,05**
75% percentile	91,53	91,72	94,31	-
Maximum	98,27	95,71	99,14	-

Discussion

Polycystic ovary syndrome is a condition which occupies a special position in the contemporary world of medicine. It affects women of childbearing age, but also influences the metabolic and hormonal status of the post-reproductive population. PCOS is a broad, interdisciplinary problem and requires multilevel research, starting from the mechanism of its pathogenesis. Recently, the topic has been dominated by much controversy and questions but certain facts have been established.

Hyperinsulinemia and insulin resistance seem to reflect the connection between ovulatory disturbances, hyperandrogenic state, and metabolic dysfunction. Unfortunately, it is not included in any of the applicable criteria defining PCOS. Insulin acts in multidirectional ways, influencing metabolic pathways, regulating hormones in numerous tissues, including the ovary. However, only a part of its action has been discovered due to the complicated receptor cross-actions and linkages. IGFs 1 and 2 are the two substances which are structurally and functionally similar to insulin.

Considerations about the pathogenesis of PCOS and the involved pathomechanisms are complicated by multiple additional symptoms such as: obesity, dyslipidemia, gonadotropin secretory dysfunctions, cardiovascular diseases, hypertension and diabetes. PCOS patients have been proven to suffer from genetic problems leading to disorders of expression and secretion of growth factors, inflammatory mediators and other molecules, which regulate steroidogenesis pathways and folliculogenesis [26].

The literature about the role of IGF-1 in follicle growth physiology, androgen synthesis, and theca-interstitial cells proliferation/apoptosis processes [17, 18, 27, 28], emphasizes the need to assess its role in the pathophysiology of PCOS.

Most authors interested in alternative splicing of the IGF-1 gene publish raw data, merely ascertaining the presence of the isoforms in various tissues, or comparing the expression levels between A, B and C fractions. Moschos et. al., found the IGF-1A isoform in the HLE-B3 cells of human lens epithelium [29]. Earlier, the IGF-1 isoforms were isolated from rat tissues: liver, muscles, lungs, testes and kidneys [30], and human tissues: hepatocytes, myocytes, myoblasts and neuroblasts [31].

IGF-1C(MGF) and/or IGF-1A have been proven to participate in the early repair process in muscle fibers following injury or loading [25]. They also demonstrate neuroprotective actions in animal studies, suggesting its possible future use for humans [32, 33, 34, 35]. As mentioned above, the literature reports that the

expression of IGF-1 isoforms varies in certain tissue states. The exact mechanisms of these actions remain to be fully elucidated.

To the best of our knowledge, there have been no data identifying IGF-1 isoforms in human ovaries. In our paper, identification of the IGF-1 isoforms in human ovary revealed that IGF-1 is produced and expressed locally, and regulation is probably driven by the autocrine and paracrine processes. IGF-1A represents the most abundant isoform, followed by IGF-1B, and then IGF-1C. The comparison between the groups, although statistically insignificant, demonstrated that the PCOS non-treated group has more expression of IGF-1A and total (IGF-1A + IGF-1B + IGF-1C), as compared to controls, and to women treated with metformin. The percentage of IGF-1A was the lowest in the PCOS M(-)group and statistically different from the control group. This might postulate the potential effect of metformin on the expression of the IGF-1 isoforms. Our findings about the differences in the expressions seem to indicate a promising scientific course but further data collection and analysis are needed to confirm that IGF-1 isoforms play a role in the pathogenesis of PCOS.

Conclusion

Our study demonstrated that gene transcripts for A, B and C isoforms of insulin-like growth factor-1 are present in human ovaries. To the best of our knowledge, our study has been the first in this field of research. In addition, in terms of the percentage of total, the IGF-1A isoform seems to be the dominant form in the ovaries of women, both healthy and diagnosed with PCOS, and its amount differs statistically between the M(-) group and controls. This is a preliminary report and the project will continue. Further studies are needed, but these findings may be the starting point for the discussion and research on the influence of locally produced isoforms of IGF-1 on the still elusive pathogenesis of PCOS.

Oświadczenie autorów:

1. Maciej Brązert – autor koncepcji i założeń pracy, zebranie i analiza materiału, analiza statystyczna wyników, przygotowanie manuskryptu i piśmiennictwa – autor zgłaszający i odpowiedzialny za manuskrypt.
2. Leszek A. Pawelczyk – autor koncepcji i założeń pracy, analizy i interpretacji wyników, przygotowanie, korekta i akceptacja ostatecznego kształtu manuskryptu.

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Autorzy nie zgłaszają konfliktu interesów oraz nie otrzymali żadnego wynagrodzenia związanego z powstawaniem pracy.

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