Biologia Serbica, 2024, 46: 89-99

DOI: 10.5281/zenodo.13925836

Original paper

Transcription of cAMP and MAPK signaling markers is disturbed in spermatozoa of aged rats

Isidora M KESELJ, Tatjana S KOSTIC, Silvana A ANDRIC*

Laboratory for Reproductive Endocrinology and Signaling, Laboratory for Chronobiology and Aging, CeRES, DBE, Faculty of Sciences, University of Novi Sad, 21000 Novi Sad, Serbia

Accepted: 10 September 2024 / Published online: 24 September 2024

Summary. According to the World Health Organization, the burden of men (in/sub)fertility remains high, increasing over the years, it is significant, likely underestimated, and has not displayed any decrease over the last 20 years. Besides, an increasing number of unexplained cases of infertile males and a reduction in the fertility rate in men younger than age 30 have been reported. This study was designed to search for the possible markers of decreased spermatozoa functionality during aging. The in vivo experimental models were the aged male rats: 12-month-old-rats (middle-aged; 5 per group), 18-month-old rats (middle-old-age; 5 per group), 22-month-old rats (old-age; 3 per group). The transcriptional profiles of 12 markers of the cAMP signaling pathway and 10 markers of the MAPK signaling pathway were followed in individual spermatozoa samples isolated from the epididymis. The results show that the transcriptions of 83.33% (10/12) cAMP signaling markers and 60% (6/10) MAPK signaling markers were changed during aging. The dominant effect on both signaling pathways was an increase in the levels of transcripts. The most prominent changes (83.33%) in cAMP signaling were observed in the oldest group (22-month-old rats), followed by 70% in 18-month-old rats (middle-old-age) and 66.67% in 12-month-old rats (middle-aged). The levels of transcript for soluble adenylyl cyclase (ADCY10), an enzyme important for capacitation and spermatozoa functionality, significantly increased in spermatozoa obtained from 12-month-old rats (middle-age) and 18-month-old rats (middle-old-age) but decreased in spermatozoa from 22-month-old rats (old-age). Similar patterns were evident following the most prominently expressed catalytic and regulatory subunits of protein kinase A (*Prkaca*, *Prkar1a*). The effects were not repeated following the MAPK signaling pathway since 60% of changes were evident in both, the youngest group (12-month-old rats) and the oldest group (22-month-old rats), while in 18-month old rats (middle-old-age) only 30% of changes were observed.

Keywords: aging, cAMP signaling markers, MAPK signaling markers, spermatozoa.

INTRODUCTION

Infertility refers to the inability to conceive a child after a year of regular, unprotected intercourse. It is estimated that about 15% of couples are affected by infertility, with male factors contributing to about 40-50% of infertility cases factors (Agarwal et al. 2021; Bardhi and Drakopoulos 2021; Wu et al. 2022; Eisenberg et al. 2023; Niederberger 2024; Wei et al. 2024).

Male infertility can be caused by various factors (Agarwal et al. 2021; Bardhi and Drakopoulos 2021; Wu et al. 2022; Eisenberg et al. 2023; Niederberger 2024; Wei et al. 2024). Some common factors associated with male sub- and infertility are a low concentration of sperm in the ejaculate, poor sperm motility, abnormal sperm morphology (Agarwal et al. 2021; Wu et al. 2022; Eisenberg et al. 2023), as well as varicocele (Jensen et al. 2017). Besides, hormonal imbalances and

E-mail: silvana.andric@dbe.uns.ac.rs

hormonal issues, such as problems and diseases related to the hypothalamus, pituitary gland, or testicles, can impact sperm production (Sengupta et al. 2021). Moreover, genetic conditions, like Klinefelter syndrome, Y chromosome microdeletions and changes in particular genes, can cause infertility in men (Krausz and Riera-Escamilla 2018; Flannigan 2020; Kyrgiafini and Mamuris 2023; Qu et al. 2023; Sha et al. 2023). Delaying parenthood, alone or in combination with unhealthy lifestyle choices, such as smoking, excessive alcohol consumption, drug use, poor diet, obesity and a sedentary lifestyle can also contribute to fertility problems (Bisht et al. 2017; Agarwal et al. 2021; Wu et al. 2022; Eisenberg et al. 2023). Sexual health issues such as erectile dysfunction or premature ejaculation can severely affect a young man's fertility. These issues may be related to physical or psychological factors related to stress, medication, and lifestyle. Sexually transmitted infections such as chlamydia or gonorrhea can cause scarring and blockages in the reproductive tract, leading to infertility in men. The same applies to certain medications, like anabolic steroids, painkillers or for cancer or chronic illnesses treatment. Besides, young men may be exposed to environmental toxins, chemicals, or radiation that can adversely effects on sperm production (Bardhi and Drakopoulos 2021; Silva et al. 2022; Eisenberg et al. 2023).

Accordingly, infertility can affect men of any age, including young men and it is extremely difficult to find the basis of the problem and to solve it. For addressing infertility in young men early diagnosis and intervention are crucial. It is important for young men to be proactive about their reproductive health, seeking medical advice and fertility assessments if conception is proving difficult. Early intervention can improve the chances of successful treatment and achieving a healthy pregnancy. Lifestyle modifications, such as maintaining a healthy diet, regular exercise, and avoiding harmful substances, can positively impact fertility. Treating underlying medical conditions, addressing hormonal imbalances, and managing stress can improve fertility outcomes (Agarwal et al. 2021; Silva et al. 2022; Taitson et al. 2022).

Aging can be defined as a time-dependent general decline in the physiological functions of the organism, associated with an increased risk of morbidity and mortality. However, the most prominent characteristics observed in cellular aging are changes in the mitochondria. Mutations in mtDNA and changes in mitochondrial proteins also progressively increase with age (Amaral and Ramalho-Santos 2009). Perturbations in mitochondrial function and defects in mitochondrial quality control systems are hallmarks of ageing and age-related pathologies (Markaki et al. 2021). Even though mitochondrial impairment is associated with aging, the high complexity of aging phenotypes, and their underlying molecular mechanisms, make deciphering the real causing elements difficult (Theurey and Pizzo 2018). Also, the function of Leydig cells declines with aging, both in rats and humans, leading to a decrease in testosterone production (Chen et al. 2020; Sokanovic et al. 2021, 2014). The impaired steroidogenic function of Leydig cells in rats is first observed around the twelfth month of life, with function progressively declining until the fifteenth month, and then maintaining at a low level from the fifteenth to the twenty-fourth month. In addition, the cAMP and MAPK signaling were disturbed in Leydig cells from old rats (Sokanovic et al. 2021, 2014). Age-dependent decreases in testosterone and the number of healthy Sertoli cells are the reasons for impaired spermatogenesis, lowered sperm counts, and worsening semen and sperm quality (Feuz et al. 2024).

The spermatozoa of aged men more frequently undergo age-related modifications, potentially leading to various consequences. The occurrence of such alterations in male germ cells has important implications since any damage to reproductive cells might produce permanent effects not only on the fertility status of the questioned patient but also on the health and viability of the offspring (Chianese et al. 2014). The aging process can affect spermatozoa through various mechanisms, leading to changes in sperm quality and function: reduced sperm production and motility, an increase in sperm with abnormal morphology, an increased risk of DNA damage and fragmentation, increased sperm DNA methylation, telomere shortening, mitochondrial dysfunction, increased reactive oxygen species (ROS). Besides, aging can lead to changes in the composition of seminal fluid, including alterations in the levels of antioxidants and other substances that influence sperm function. It is important to note that the impact of aging on male fertility can vary among individuals. While advanced paternal age is associated with certain risks, many older men can still father healthy children. However, understanding these mechanisms helps researchers and healthcare professionals explore potential interventions and treatments related to infertility in men (Agarwal et al. 2021; Bardhi and Drakopoulos 2021; Silva et al. 2022; Taitson et al. 2022; Eisenberg et al. 2023).

Accordingly, in search for possible reasons for male (in/sub)fertility, we hypothesized that the transcription patterns of the elements of the two most important signaling pathways for spermatozoa homeostasis and functionality, the AMP and the MAPK signaling pathways, are changed during aging and that we can apply that knowledge in future research on young (in/sub)fertile males. The rationale for using a model of aged animals was believing that similar transcription patterns of the AMP and the MAPK signaling elements exist in spermatozoa of young individuals with fertility problems. The in vivo experimental models of the aged male rats: 12-month-old-rats (middle-aged), 18-month-

old-rats (middle-old-age) and 22-month-old-rats (old-age). The transcriptional profiles of the elements of the two most important signaling pathways for spermatozoa homeostasis, the AMP and the MAPK signaling pathways were followed in spermatozoa isolated from the epididymis.

MATERIALS AND METHODS

All samples, commercial reagents, primers, and software that were used in this study are given in Table 1.

All experiments were performed in the Laboratory for Reproductive Endocrinology and Signaling and Laboratory for Chronobiology and Aging, Faculty of Sciences at the University of Novi Sad (*https://wwwold.dbe.pmf.uns.ac.rs/en/nauka-eng/lares*). All the methods used in this study have been previously reported by our group (for all references please see Sokanovic et al. 2014; Starovlah et al., 2022a, 2022b) and follow the relevant guidelines and regulations.

A statement that the authors complied with ARRIVE guidelines and institutional animal care and use committee guidelines

The authors complied with ARRIVE guidelines, and all experiments were in adherence to the ARRIVE guidelines. Furthermore, all experimental protocols were approved (statement no. 04-81/114, dated 25 September 2020) by the local Ethical Committee on Animal Care and Use of the University of Novi Sad operating under the rules of the National Council for Animal Welfare and the National Law for Animal Welfare (copyright March 2009), following the NRC publication "Guide for the Care and Use of Laboratory Animals" and NIH's "Guide for the Care and Use of Laboratory Animals".

Animals and experimental models

All the experiments were performed using male *Wistar* rats which were bred and raised in the animal facility of the Faculty of Sciences University of Novi Sad. The animals were raised under controlled environmental conditions (22 ± 2 °C; 14 h light and 10 h dark cycle) with food and water available ad libitum.

The in vivo experimental model of aging and the selection of the periods of aging were previously described by our group (Sokanovic et al. 2014). It was demonstrated that during the aging of *Wistar* rats, more than a 21% reduction in serum testosterone concentrations was detected in the 12-month-old and an additional 50% in 15th to 24-month-old rats. The model of aging included adult 3-month-old male rats (13 rats), middle-aged 12-month-old (5 rats), middle-old-age 18-month-old (5 rats) and old-age 22-month-old (3 rats) male rats that were undisturbed during their life period (Fig. 1). At the end of the experimental period, all

Table 1. Key resources table.

Resource or reagent	Source	Identifier
Experimental model and biological samples		
Wistar rat	LaRES and ChronAge Laboratories (DBE, Faculty of Sciences, University of Novi Sad, Serbia)	*http://wwwold.dbe.pmf.uns.ac.rs/en/nauka-eng/lares*
Spermatozoa	3-,12-,18-,22-month-old male rats	NA
Commercial Reagents/Assays		
GenElute Mammalian Total RNA Miniprep Kit	Merck, Darmstadt, Germany,	*www.sigmaaldrich.com*
DNase I (RNase-free) treatment	New England Biolabs, USA	*https://international.neb.com*
High-Capacity Kit for cDNA	Applied Biosystems/Thermo Fisher Scientific, USA	*https://www.thermofisher.com*
Power SYBR® Green PCR Master Mix	Applied Biosystems/Thermo Fisher Scientific, USA	*https://www.thermofisher.com*
Primers		
Tables 2 and 3	This paper	*www.ncbi.nlm.nih.gov/sites/entrez*
Software		
GraphPad Prism 8 Software	GraphPad Prism	*https://www.graphpad.com/scientific-software/prism*

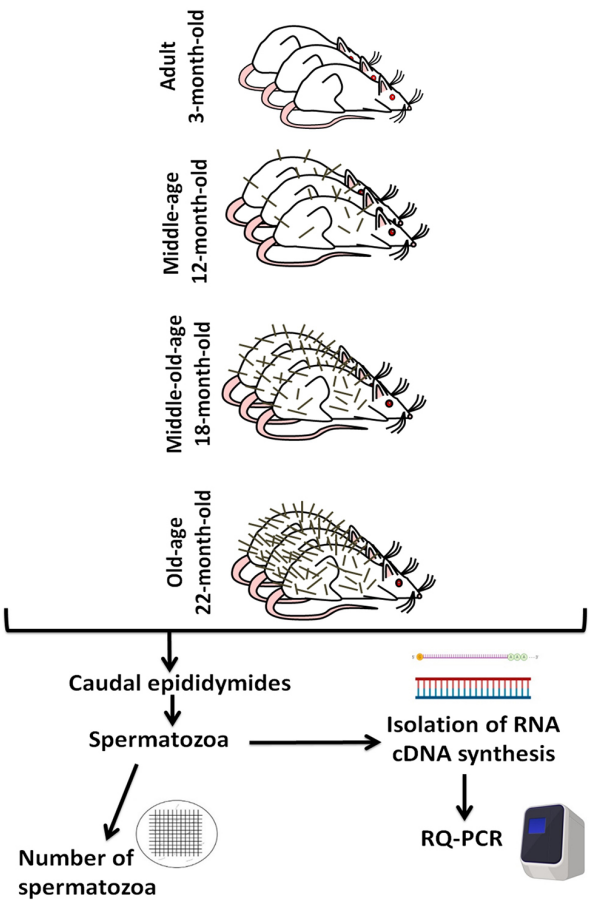

Fig. 1. Experimental model of aging used to assess transcriptional profiles of cAMP and MAPK signaling pathway markers.

animals were quickly decapitated without anesthesia. Animals were dissected and inspected, and only those without obvious morphological changes and normal caudal epididymides structure were used. In each experiment, animals of different age categories consisted of two to seven animals. The experiment was repeated twice.

Spermatozoa isolation

Isolation of spermatozoa from the caudal epididymides was carried out following the WHO laboratory manual (*https://www.who.int/publications/i/item/9789240030787*) with modifications for rat spermatozoa isolation, previously described by our group (Starovlah et al. 2021, 2022a, 2022b). In short, caudal epididymides were quickly isolated, and the surrounding adipose tissue was removed. Isolated epididymides were placed in a petri dish containing medium for isolation and preservation of spermatozoa (1% M199 in HBSS with 20 mM HEPES buffer and 5% BSA). Epididymides were finely punctuated with a 25G needle to enable spermatozoa to be released into the medium and incubated at 37 °C for 10 min. Released spermatozoa were collected and centrifuged for 5 min at 700*g* at room temperature. The supernatant was removed, and the pellet was resuspended in the medium. The number of isolated spermatozoa was calculated using a Makler counting chamber (Sefi-Medical Instruments, Ltd, Israel). Isolated spermatozoa were stored at -70 °C, before RNA isolation and the subsequent gene transcription analysis.

Isolation of RNA and cDNA synthesis

Spermatozoa isolated from the caudal epididymides were stored at -70 °C until they were used for the isolation of total RNA. Total RNA isolation was performed using the GenElute Mammalian Total RNA Miniprep Kit according to the protocol recommended by the manufacturer (Merck, Darmstadt, Germany, *www.sigmaaldrich.com*), followed by the DNase I (RNase-free) treatment (New England Biolabs, MA, USA, *www.neb.com*). The concentration and purity of isolated total RNA were measured using the BioSpec nano-spectrophotometer (Shimadzu, Japan, *www.shimadzu.com*). Furthermore, the first-strand cDNA was synthesized using the High-Capacity Kit for cDNA preparation following the manufacturer's protocol (Thermo Fisher Scientific, MA, USA, *www.thermofisher.com*). The difference in concentration of RNA, and further cDNA, between samples was corrected using control primers for *Gapdh*, as described previously by our group (for reference, please see Starovlah et al. 2022a, 2022b).

Relative quantification of gene expression

Relative quantification of gene expression was done using real-time PCR and was quantified by SYBR®Green-based chemistry from Applied Biosystems (Thermo Fisher Scientific, MA, USA, *www.thermofisher.com*). For each reaction, 15 ng of cDNA was used (calculated from starting RNA) in the volume of 2.5 μL with specific primers at a final concentration of 500 nM. Primer sequences used for real-time PCR analysis and Ct values, as well as GenBank accession codes for full gene sequences (*www.ncbi.nlm.nih.gov*), are given in Tables 2–3. Relative gene expression quantification of *Gapdh* was measured in each sample and used to correct the variations in cDNA concentrations between samples. Relative quantification of each gene was performed in duplicate, two times, for each independent in vivo experiment. The real-time PCR reactions were carried out in the Eppendorf Mastercycler ep realplex 4 and post-run analyses were performed using Mastercycler® ep realplex software (for reference, please see Starovlah et al. 2022a, 2022b).

Table 2. Primers sequences used for the real-time PCR analysis of cAMP signaling elements.

Gene	Accession code	Primers	Primer length	Product length	AV Ct
Adcy3	NM_130779	F: 5'-GCATCGAAACCTACCTCATCA-3' R: 5'-TGGGCTCCTTGGTCTCA ATAA-3'	21 bp 21 bp	141 bp	31.17
Adcy5	NM_022600	F: 5'-AACCAGGTGAACGCATGTCA-3' R: 5'-CTCTGGGAAGTTGCAGTTGGA-3'	20 bp 21 bp	105 bp	30.22
Adcy6	NM_012821	F: 5'-CTGCCTCAGCCTGCTTATGTG-3' R: 5'-GGAGTCCTGGCGGAAGCT-3'	21 bp 18 bp	99 bp	27.14
Adcy7	NM_053396	F: 5'-TTCCGTGCGTGTAACCCGCT-3' R: 5'-GCCTTCTGCCTCCGTCCGTT-3'	20 bp 20 bp	123 bp	26.94
Adcy8	NM_017142	F: 5'-ATTGCCTCAGTGGTGACTA-3' R: 5'-CAAACTCTCCTCGGGCT-3'	19 bp 17 bp	113 bp	32.85
Adcy9	NM_001106980	F: 5'-TCACCAAGCTGTACGCCCGG-3' R: 5'-GGGCTGTCAACACGTCCCGA-3'	20 bp 20 bp	124 bp	29.52
Adcy10	NM_021684	F: 5'-CCAGGCATCGTGACCTGCGA-3' R: 5'-ACTGGTCCGGGATCCGCAAC-3'	20 bp 20 bp	113 bp	30.77
Prkaca	NM_001100922.1	F: 5'-TCAGTGAGCCCCACGCCCGTT-3' R: 5'-TCTCGGGCTTCAGGTCCCGG-3'	21 bp 20 bp	99 bp	26.30
Prkacb	NM_001077645	F: 5'-GGGTCATGGGGAACACGGCG-3' R: 5'-CCAGCATTACTCGGGGGAGGGT-3'	20 bp 22 bp	124 bp	26.95
Prkar1a	NM_013181	F: 5'-TGTGCTGCAGCGTCGGTCAG-3' R: 5'-AGTGGCAGCCCGAGGACGAT-3'	20 bp 20 bp	112 bp	25.05
Prkar2a	NM_019264	F: 5'-GCCCGACCTCGTCGACTTCG-3' R: 5'-TCCTGCGCGTGAAAGGTCGT-3'	20 bp 20 bp	108 bp	27.08
Prkar2b	NM_001030020	F: 5'-CCCATGCGCTCCGATTCCGA-3' R:5'-GCACATACCGAGGCACGCCT-3'	20 bp 20 bp	107 bp	29.64
Gapdh	NM_017008	F: 5'-TGCCAAGTATGATGACATCAAGAAG-3' R: 5'-AGCCCAGGATGCCCTTTAGT-3'	25 bp 20 bp	110 bp	21.62

Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from NCBI Entrez Nucleotide database (*www.ncbi.nlm.nih.gov/sites/entrez*). F - forward; R - reverse.

Statistical analysis

Results of the experiments are represented by group means ± SEM values of the individual variation from two independent experiments. Results from each experiment were analyzed by one-way ANOVA, followed by the Student–Newman–Keuls multiple range test. All the statistical analyses were performed using GraphPad Prism 8 Software (GraphPad Software 287 Inc., La Jolla, CA, USA). In all cases, a p-value <0.05 was statistically significant.

RESULTS

To understand the influence of aging on the transcriptional profile of main markers of cAMP and MAPK signaling pathways, important for spermatozoa functionality, four different age categories were used. In this research, we used adult (3-month-old), middle-age (12-month-old), middle-old-age (18-month-old) and old-age (22-month-old) rats. After the experimental period, transcriptional profiles of markers of cAMP and MAPK signaling pathways were obtained in spermatozoa isolated from caudal epididymides.

Transcriptional profiles of markers associated with the cAMP signaling pathway were changed in spermatozoa of aged rats

The analysis of the relative expression of the main markers of the cAMP signaling pathway showed a disturbed expression in spermatozoa of 12-month-old, 18-month-old

Table 3. Primers sequences used for the real-time PCR analysis of MAPK signaling elements.

Gene	Accession code	Primers	Primer length	Product length	AV Ct
Mapk1	NM_053842.1	F: 5'-GTTCTGCACCGTGACCTCAAG-3' R: 5'-GCAAGGCCAAAGTCACAGATC-3'	21 bp 21 bp	80 bp	25.38
Mapk3	NM_017347.2	F: 5'-TCCCTCTCAAGCTGCCACAT-3' R: 5'-ACATCCAATCACCCACACACA-3'	20 bp 21 bp	60 bp	26.79
Mapk6	NM_031622.2	F: 5'-CATTTGAACTGGCATGTCGTTT-3' R: 5'-CCTGCACTGCATTGTTTTGC-3'	22 bp 20 bp	62 bp	25.74
Mapk7	NM_001191547.1	F: 5'-GCCCCTTCCACTAGCCTTTT-3' R: 5'-GAACCAGGCAACCCACTAGGT-3'	20 bp 21 bp	62 bp	26.97
Mapk8	NM_053829.2	F: 5'-TCAACGTCTGGTATGATCCTTCA-3' R: 5'- CTGCTTGTCAGGGATCTTTGG-3'	23 bp 21 bp	62 bp	27.03
Mapk9	NM_017322.1 NM_001270544.1 NM_001270545.1	F: 5'-GGAAGGCTGCCGATGAAA-3' R: 5'-AGCCAGAGTCCTTCACAGACAAG-3'	18 bp 23 bp	57 bp	26.53
Mapk11	NM_001109532.2	F: 5'-GGGCGCTGACCTGAATAACA-3' R: 5'-GCAGCAGCTGGTAGACAAGGA-3'	20 bp 21 bp	80 bp	32.23
Mapk12	NM_021746.1	F: 5'-GGATGTGTTCACTCCCGATGA-3' R: 5'-CCAGGTCAGTGCCCATGAAT-3'	21 bp 20 bp	80 bp	27.53
Mapk13	NM_019231.2	F: 5'-CTGGTCTGTTGGCTGCATCA-3' R: 5'-TCAGCTGGTCCAGGTAGTCCTT-3'	20 bp 22 bp	80 bp	26.90
Mapk14	NM_031020.2	F: 5'-GCTGTCGACCTGCTGGAAAA-3' R: 5'- TAGGCATGCGCAAGAGCTT-3'	20 bp 19 bp	80 bp	25.30
Gapdh	NM_017008	F: 5'-TGCCAAGTATGATGACATCAAGAAG-3' R: 5'-AGCCCAGGATGCCCTTTAGT-3'	25 bp 20 bp	110 bp	21.62

Primers were designed by using software Primer Express 3.0 (Applied Biosystems) and full genes sequences from NCBI Entrez Nucleotide database (*www.ncbi.nlm.nih.gov/sites/entrez*). F - forward; R - reverse.

and 22-month-old animals compared to 3-month-old, adult, animals (Fig. 2). Transcriptional levels of five out of six analyzed adenylyl cyclases (83%) were disturbed in spermatozoa of rats of different age category. The level of *Adcy3* was significantly increased in spermatozoa from 12-month-old rats (1.51-fold) and 18-month-old group (1.63-fold) compared to the samples of spermatozoa obtained from the adult (3-month-old) group, while decreased in spermatozoa from 22-month-old (2.22-fold) compared to 12-month-old group. Increased transcription of *Adcy6* was detected in spermatozoa obtained from all analyzed aged groups, 12-month-old (2.13-fold), 18-month-old (1.66-fold) and 22-month-old (1.73-fold) compared to the adult group of 3-month-old animals. On the other hand, decreased transcription of *Adcy7* was observed only in spermatozoa of 22-month-old animals (1.85-fold). The level of *Adcy8* was significantly increased in 12-month-old (2.53-fold), 18-month-old (5.83-fold) and 22-month-old (4.42-fold). The level of *Adcy9* was decreased in 18-month-old (1.66-fold) and 22-month-group (1.99-fold) compared to the adult group of 3-month-old animals. Transcription of *Adcy10,* an enzyme important for capacitation and spermatozoa functionality, was increased in spermatozoa of 12-month-group (12.65-fold) and 18-month-old animals (8.64-fold) but decreased in spermatozoa of 22-month-old animals (1.76-fold vs. 3-month-old and 22.22-fold vs. 12-month-old).

In addition to the transcripts for enzymes producing cAMP, the transcripts for subunits of the downstream target of cAMP, protein kinase A (PRKA), were followed. The transcriptional profile of catalytic subunit alpha of the PRKA (*Prkaca*) had a similar transcriptional profile of increase in spermatozoa obtained from 12-month-old rats (1.59-fold) and 18-month-old rats (1.68-fold) and decreased (1.79-fold) transcription in spermatozoa from 22-month-old rats. On the other hand, the transcript for regulatory subunit alpha type 1 of the PRKA (*Prkar1a*) was increased (1.61-fold)

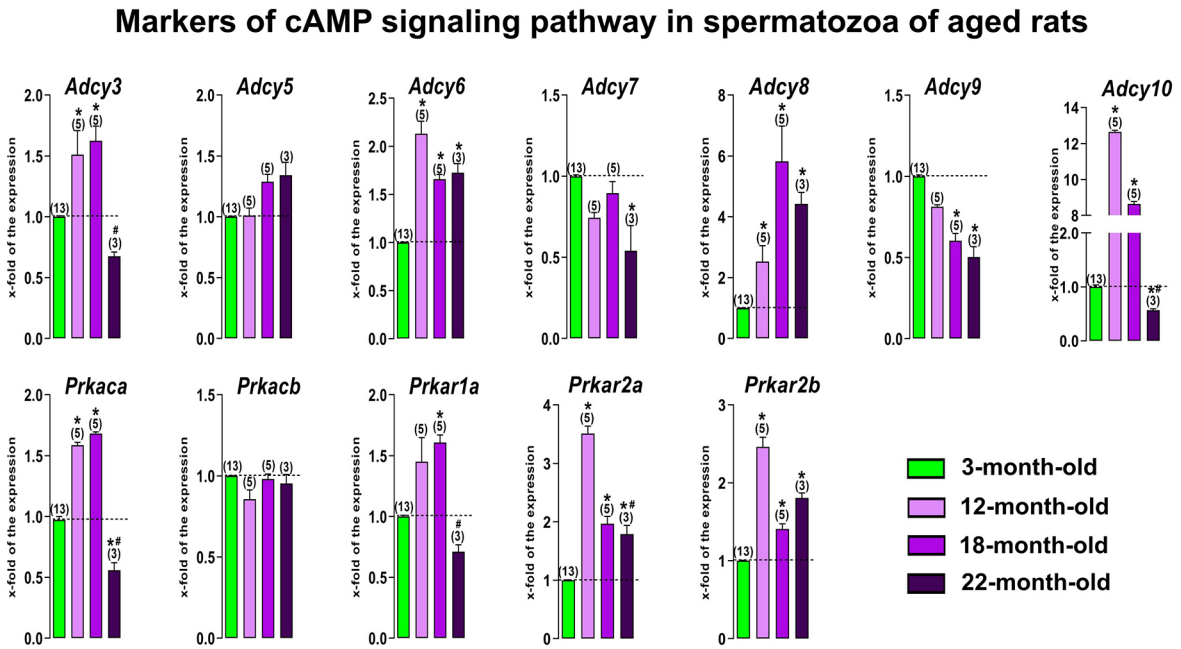

Fig. 2. Transcription of cAMP signaling pathway markers is significantly changed in spermatozoa of aged rats.
Spermatozoa were isolated from undisturbed middle-age (12-month-old), middle-old-age (18-month-old) and old-age (22-month-old) rats, as well as from control adult (3-month-old) rats. Spermatozoa was further used for RNA isolation followed by analysis of the transcriptional profile of main markers of cAMP signaling pathways. Data bars represent mean ± SEM values of two independent in vivo experiments. Number in brackets above the bar represents number of animals used for the analysis. Statistical significance was set at level $p < 0.05$: * vs. control group, and # vs. 12-month-old group.

only in spermatozoa obtained from 18-month-old rats. The other analyzed regulatory subunits of the PRKA (*Prkar2a* and *Prkar2b*) showed increased transcriptional profiles in all analyzed aged groups compared to adults, 3-month-old group. *Prkar2a* was increased in 12-month-old rats (3.51-fold), 18-month-old rats (1.97-fold) and 22-month-old rats (1.79-fold). In the same samples of spermatozoa, the transcript for *Prkar2b* was also increased: in 12-month-old rats (2.46-fold), 18-month-old rats (1.41-fold) and 22-month-old group (1.81-fold) compared to adults (3-month-old rats).

Transcriptional profiles of markers associated with the MAPK signaling pathway were changed in spermatozoa of aged rats

Markers of MAPK signaling pathway, important for spermatozoa functionality and male fertility, show changed transcriptional profile of six out of ten (60%) analyzed markers in spermatozoa of aged rats, compared to an adult, 3-month-old rats (Fig. 3). Level of Mapk3 was significantly increased in spermatozoa of 12-month-old rats (1.69-fold), while decreased in spermatozoa of 22-month-old rats (2.10-fold vs. 3-month-old and 3.48-fold vs. 12-month-old). The same transcriptional profile was observed in the level of Mapk6, with increased transcription in spermatozoa of 12-month-old rats (1.76-fold), while decreased in spermatozoa of 22-month-old rats (1.76-fold vs. 3-month-old and 3.10-fold vs. 12-month-old). The transcriptional profile of Mapk7 in spermatozoa of 12-month-old rats was increased (1.66-fold), as well as in the 18-month-old group (1.51-fold), but decreased in the 22-month-old group (1.54-fold vs. control, and 2.57-fold vs. 12-month-old). Transcription of Mapk8 was increased in spermatozoa of 12-month-old (2.92-fold), 18-month-old (1.73-fold) and 22-month-old (1.43-fold) compared to control (3-month-old) rats, while decreased in 22-month-old (2.04-fold) compared to the 12-month-old group. Increased transcription of Mapk11 was observed in 12-month-old (3.08-fold) and 22-month-old (2.94-fold) rats, while transcription of Mapk13 was increased in all analyzed aged rats, 12- month-old (3.99-fold), 18- month-old (2.75-fold) and 22-month-old (2.17-fold).

DISCUSSION

According to the World Health Organization, the burden of (in/sub)fertility in men remains high and it is increasing over the years. Besides, it is significant, likely underestimated, and has not displayed any decrease over the last

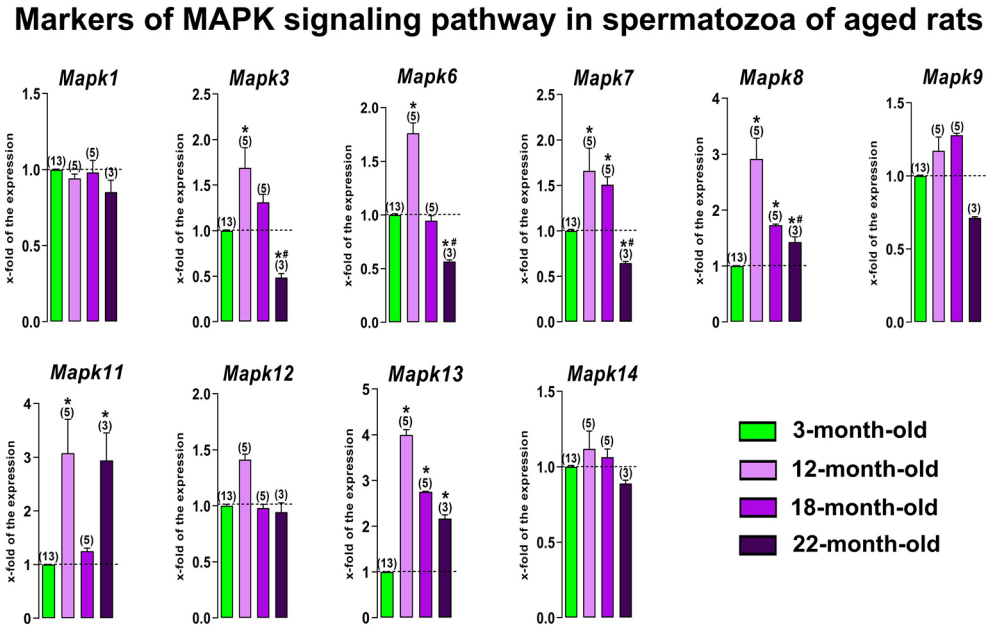

Fig. 3. Transcriptional profiles of MAPK signaling pathway markers in spermatozoa of aged rats.
Spermatozoa were isolated from undisturbed middle-age (12-month-old), middle-old-age (18-month-old) and old-age (22-month-old) rats, as well as from control adult (3-month-old) rats**.** Spermatozoa was further used for RNA isolation followed by analysis of the transcriptional profile of main markers of MAPK signaling pathways. Data bars represent mean ± SEM values of two independent in vivo experiments. Number in brackets above the bar represents number of animals (3-13) used for the analysis. Statistical significance was set at level $p < 0.05$: * vs. control group, and # vs. 12-month-old group.

20 years. Moreover, an increasing number of unexplained cases of infertile males and a decrease in the fertility rate in men younger than age 30 have been reported. Infertility can affect men of any age and it is extremely difficult to find the basis of the (in/sub)fertility. However, understanding these mechanisms helps researchers and healthcare professionals to explore potential interventions and treatments of the (in/sub)fertility in men (Agarwal et al. 2021; Bardhi and Drakopoulos 2021; Wu et al. 2022; Eisenberg et al. 2023; Niederberger 2024; Wei et al. 2024).

In search for the possible markers of decreased spermatozoa functionality during aging and to suggest those markers as eventual predictors of (in/sub)fertility we designed the in vivo experiments on the aged male rats: 12-month-old-rats (middle-aged), 18-month-old-rats (middle-old-age), 22-month-old-rats (old-age). The target molecules in this study were the elements of the two most important signaling pathways for spermatozoa homeostasis, the AMP and the MAPK signaling pathways, since both signaling pathways are essential for spermatozoa function and homeostasis (Finkelstein et al. 2020; Starovlah et al. 2022b, 2022a).

cAMP is a secondary messenger produced by enzymatic activities of adenylyl cyclases (ADCYs) and degraded by enzymatic activities of phosphodiesterases (PDEs). cAMP signaling plays a crucial role in various cellular processes (Zaccolo et al. 2021; Bock et al. 2024), including those involved in spermatozoa (Zaccolo et al. 2021; Bock et al. 2024). cAMP signaling is important for spermatozoa homeostasis as a mechanism to regulate various functions related to sperm maturation, motility, and fertilization. Moreover, cAMP signaling is involved in the regulation of capacitation, and changes in intracellular cAMP levels are associated with capacitation events. Besides, cAMP is a key regulator of sperm motility and high levels of cAMP are often associated with increased sperm motility since cAMP helps regulate the activity of proteins and enzymes that influence the movement of the sperm tail (Schuh et al. 2006; Wertheimer et al. 2013; Orta et al. 2018). Moreover, cAMP signaling is involved in the regulation of the acrosome reaction and release of enzymes, allowing the spermatozoa to penetrate the protective layers of the oocyte and facilitate fertilization. cAMP signaling is involved in the regulation of proteins and processes that facilitate the interaction between spermatozoa and oocytes (Schuh et al. 2006; Wertheimer et al. 2013). Understanding the intricate details of cAMP signaling in spermatozoa involves the study of specific proteins, receptors, and downstream effectors that mediate these processes. Researchers continue to explore these mechanisms to gain

insights into fertility and potential targets for therapeutic interventions (Schuh et al. 2006; Wertheimer et al. 2013; Orta et al. 2018; Starovlah et al. 2022b, 2022a). The results of our study presented here show that the transcriptions of 83.33% (10/12) cAMP signaling markers were altered in spermatozoa of rats in different age categories. The dominant effect of aging on transcription of cAMP signaling markers in spermatozoa was an increase in the levels of transcripts. The most prominent changes (83.33%) in cAMP signaling were observed in the oldest group (22-month-old-rats), followed by 70% in 18-month-old-rats (middle-old-age) and 66.67% in 12-month-old-rats (middle-aged) suggesting that aging promotes alterations in the cAMP signaling. The levels of transcript for soluble adenylyl cyclase (ADCY10), an enzyme related to capacitation and spermatozoa functionality (Akbari et al. 2019), significantly increased in spermatozoa obtained from 12-month-old-rats (middle-age) and 18-month-old-rats (middle-old-age) but decreased in spermatozoa from 22-month-old-rats (old-age). A graduate increase of ADCY10 was observed in Leydig cells isolated from the same rats (Sokanovic et al. 2014). Since the bicarbonate-responsive ADCY10 is a regulator of pH homeostasis, a coupler for various metabolic processes to cAMP signaling, and a candidate for metabolic sensor via cAMP signaling (Chang and Oude-Elferink 2014) it could be an important link between cAMP signaling and metabolic changes in spermatozoa during aging. Similar patterns to ADCY10 were evident following the most prominently expressed catalytic and regulatory subunits of protein kinase A (*Prkaca, Prkar1a*). Eventual consequences of the increased expression of transcripts could be restored spermatozoa functionality since it was shown that cAMP signaling improves sperm motility (Schuh et al. 2006; Wertheimer et al. 2013) and it is very important for activation of CatSper channels (Orta et al. 2018). Besides, increased expressions of transcripts for the most prominently expressed catalytic and regulatory subunits of protein kinase A (*Prkaca, Prkar1a*) as well as *Prkar2a* and *Prkar2b* could be a possible adaptive and ameliorative mechanism(s) since it was shown that the PRKAR2A reduction in asthenozoospermic patients decreases sperm quality (Capkova et al. 2016), while *Prkar2b* is sensitive to heat (Yadav et al. 2018). Altogether, the markers of cAMP signaling could be promising targets for a future investigation related to male (in/sub) fertility.

Mitogen-activated protein kinase (MAPK) signaling is an important and intriguing signaling pathway that regulates various cellular processes, including spermatozoa homeostasis. MAPK signaling plays a role in regulating several key events associated with spermatozoa function including the process of spermatozoa capacitation and acrosome reaction (particularly p38 MAPK), spermatozoa motility, sperm-oocyte fusion, DNA condensation and changes in nuclear morphology. Besides, MAPK signaling can act as a mediator in the response to external factors, such as oxidative and cellular stress (Almog and Naor 2008; Rahamim Ben-Navi et al. 2016). Research in this area is ongoing, and further investigations are needed to fully understand the intricacies of MAPK signaling in spermatozoa. Additionally, dysregulation of MAPK signaling in spermatozoa has been associated with male (in/sub)fertility, making it an area of interest for both basic reproductive biology and clinical studies (Almog and Naor 2008; Rahamim Ben-Navi et al. 2016; Starovlah et al. 2022a, 2022b). The results of our study presented here show that the transcriptions of 60% (6/10) MAPK signaling markers were changed during aging. Oppositely to the effects of aging on cAMP signaling markers in spermatozoa, the response was not gradually related to age since 60% of changes were evident in both, the youngest group (12-month-old rats) and the oldest group (22-month-old rats), while in 18-month-old rats (middle-old-age) only 30% of changes were observed. Some of the increased expression of the transcripts could be compared with findings that testicular hyperthermia induces both MAPK1/3 and MAPK14 (Jia et al. 2009) and that MEK1/2 and ERK2 regulate the spermatozoa capacitation (Salgado-Lucio et al. 2020). Besides, the significant increase of *Mapk8* transcripts in all aged groups could qualify this signaling molecule as a potential negative marker since it was reported that phosphorylation of MAPK8 is associated with germ cell apoptosis and redistribution of the Bcl2-modifying factor (Show et al. 2008). Accordingly, elements of the MAPK signaling pathway in spermatozoa could serve as promising marker(s) for a future investigation related to (in/sub)fertility in men.

It is very difficult to give explanations for these effects since all signaling molecules are very well known as the essential regulators not only of the spermatozoa number/functionality (Silva et al. 2015), but also the regulators of PGC1, the biogenesis of OXPHOS, mitofusion, mitofission, and mitophagy (Dominy and Puigserver 2013; Pyakurel et al. 2015; Markaki and Tavernarakis 2020). Besides, all affected molecules in cAMP and MAPK signaling pathways are part of the complex signaling network in spermatozoa precisely regulated to provide fertility homeostasis in health and diseases (Finkelstein et al. 2020). Moreover, the physiological role of the negative crosstalk between the cAMP-PRKA-AKAP4 and the PKC-ERK1/2 pathways in the regulation of capacitation and acrosome reaction has been reported (Rahamim Ben-Navi et al. 2016).

In summary, the results show that the transcriptions of 83.33% cAMP signaling markers and 60%) MAPK signaling markers were changed during aging. The dominant effect on both signaling pathways was an increase in the levels of

the transcripts. The most prominent changes were observed in the levels of two enzymes important for capacitation and spermatozoa functionality (*Adcy10*, *Mapk11*). The results could be the basis to searching for markers of male (in/sub) fertility.

ACKNOWLEDGMENTS

This work was supported by grants from the Serbian Ministry of Education, Science and Technological Development (CIV-CeRES-2023; 451-03-9/2021-14/200125), the Autonomous Province of Vojvodina (3502), the ANDRONET action.

Data availability statement

All relevant data and samples are available from the corresponding author on request. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Silvana Andric (silvana.andric@dbe.uns.ac.rs).

Author's contributions statement

IMK – acquisition of the data; analysis and interpretation of the data; drafting the manuscript; revising manuscript critically for important intellectual content; final approval of the version to be submitted; TSK – acquisition of the data; analysis and interpretation of the data; revising manuscript critically for important intellectual content; final approval of the version to be submitted; SAA – the conception and design of the research; acquisition of the data; analysis and interpretation of the data; drafting the manuscript; revising manuscript critically for important intellectual content; final approval of the version to be submitted. All authors - approved the final version of the manuscript; agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; qualify for authorship, and all those who qualify for authorship are listed.

Conflict of interest statement

The authors declare no competing interests.

Funding

This research was funded by the Serbian Ministry of Education, Science and Technological Development (CIV-CeRES-2023, 451-03-9/2021-14/200125) and the Autonomous Province of Vojvodina (3502).

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