Original Article

Dynamic Expression and Chromatin Incorporation of ACT and CREM Transcription Factors in Testis Tissues of Infertile Men

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Abstract

Objective: Activator of CREM in the testis (ACT) is a tissue specific transcription factor which activates cAMP responsive element modulator (CREM), a key transcription factor in differentiation of round spermatids into mature spermatozoa. They bind to CRE region in the promoters of transition protein genes (*TNP1, TNP2)* and protamine genes (*PRM1* and *PRM2*), which are essential for sperm chromatin compaction, and regulates their transcription. This study was conducted to consider the expression of *ACT* and *CREM* and their regulatory roles on the expression of *PRM1, PRM2*, *TNP1* and *TNP2* genes in testis tissues of infertile men.

Materials and Methods: In this case-control study, testicular biopsies were collected from 40 infertile men and classified into three groups: obstructive azoospermia (OA, n=10, positive control), round spermatid maturation arrest (SMA, n=20), Sertoli cell-only syndrome (SCOS, n=10, negative control group). Using quantitative real-time polymerase chain reaction (PCR), the expression profile of *ACT*, *CREM*, *TNP1*, *TNP2*, *PRM1* and *PRM2* genes were assessed in testicular samples and incorporation of ACT and CREM proteins on the promoters of *PRM1, PRM2*, *TNP1* and *TNP2* genes were also evaluated by ChIP-real time PCR.

Results: Our results demonstrated significant decrease in the expression levels of *ACT*, *CREM* and in their incorporations on their target genes in SMA group in comparison to control groups (P≤0.05).

Conclusion: These data confirm that there is low expression and incorporation of ACT and CREM and of their target genes in infertilities which are associated with post-meiotic arrest.

Keywords: Activator of CREM in The Testis, cAMP Responsive Element Modulator Chromatin, Male Infertility, Spermatogenesis

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Introduction

About 15% of couples struggle with infertility and are unable to conceive and male factor is the reason for infertility in half of the cases (1, 2). Genetic factors have an important part in causing male infertility by affecting various physiological processes, specifically spermatogenesis (3, 4). Spermatogenesis is the differentiation process in which diploid spermatogonia, successively undergo mitotic, meiotic and post-meiotic stages, eventually differentiating into haploid mature spermatozoa (5).

Chromatin re-organization and condensation associated with the maturation of the paternal genome is a unique process, required for the generation of healthy functional male germ cells (6). During this process, core histones are hyperacetylated and replaced by germ cells specific proteins including transition proteins (TNP1 and TNP2) and protamines (PRM1 and PRM2) (5, 7, 8). The latter remain tightly associated with a highly condensed paternal genome within the mature spermatozoa (9, 10). Inadequate expression of these nucleoproteins can obstruct process of nuclear compaction which can ultimately lead to impaired spermatogenesis (11, 12). It was demonstrated that disruption of both *TNP1* and *TNP2* in mice led to abnormalities in sperm morphology and spermiogenesis, whereas, either *PRM1* knockout or *PRM2* knockout can cause male infertility (13, 14). cAMP responsive element modulator (CREM) and its cofactor activator of CREM in the testis (ACT) are transcription factors which bind to the cAMP response element (CRE) regions of *PRM1, PRM2*, *TNP1* and *TNP2* and increase the expression of aforementioned genes (15, 16).

CCREM protein is a member of basic domain-leucine zipper family which functions as transcription factor and binds to a palindromic sequence named CRE and initiates the transcription of its downstream genes: *TNP1, TNP2, PRM1, PRM2* (16). ACT protein is a member of Lim-only family with two zinc finger domains which binds to CREM and strengthens its function in transcribing their target genes (17). How CREM and ACT activate gene transcription can be different based on the type of the cell, in somatic cells, CREM gets phosphorylated at the Ser-117 residue and only then it binds to a CRE and triggers gene transcription whereas in the haploid cells of the testis, CREM no longer needs to be phosphorylated in order to function after binding to ACT which binds with high affinity to CREM protein (18, 19).

Studies have shown that CREM and its cofactor, ACT, have important impact on male infertility (17, 20). A study demonstrated that mice which lacked *CREM* gene had lower sperm concentration, abnormal sperm morphology and most importantly they were infertile. Another study showed that mice lacking *ACT* gene had significantly decreased sperm concentration and also they had folded or bent sperm tail, interestingly, they were still fertile (21, 22). These finding along with the fact that the expression of these two proteins are in line with each other , simply indicates the importance of ACT and CREM to male fertility (20).

The current study is aimed to consider the expression levels of *CREM* and of its cofactor *ACT* and of their target genes, encoding *TNP*s and *PRM*s, in testis tissues of infertile men in order to gain more insight into the molecular characterization of the defects observed in infertile patients with post-meiotic arrests.

Materials and Methods

Subjects

In this case-control study, 40 testicular biopsy specimens were collected from infertile male patients who were referred to Royan Institute. The samples were categorized as follows: obstructive azoospermia (OA, positive control), round spermatid maturation arrest (SMA), and Sertoli cell-only syndrome (SCOS, negative control group). The clinical features of the groups including patients' karyotype, age, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone hormone are monitored in the study. In order to obtain sperm for intracytoplasmic sperm injection (ICSI), SMA samples were obtained from TESE operation and OA samples and SMA samples were retrieved from testes with microTESE. The current research was approved by the Reproductive Biomedicine Research Center and Ethics Committee of Royan Institute (EC/91/1046) and written informed consent was obtained from all patients which allowed us to use their tissue samples.

DNA synthesis and Real-time quantitative polymerase chain reaction

RNA extraction from tissue samples was carried out with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Samples were treated with DNase-I so that DNA contamination was removed, complementary DNA strand was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. All samples were normalized with the expression of *GAPDH* gene.

The cDNA samples were quantified with quantitative reverse transcription polymerase chain reaction (qRT-PCR) using SYBR Green PCR master mix (Applied Biosystems) on a Step One Plus Real-Time PCR System (Applied Biosystem) and with designed primers which are shown in Table 1 in three groups: OA (n=10), SMA (n=20), SCOS (n=10).

The qPCR reaction for *ACT* and *CREM* genes were carried out with the following profile: 20 µL volume, containing 2 µl of template cDNA (12.5 ng/µL), 1 µl of each 5 pmol/µl primer (Sinaclone, Iran), 10 µl of SYBR Green PCR master mix and 6 µl dH_2O and followed by initial denaturation at 95°C for 4 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 1 minute.

Two replicates were carried out for each sample and gene and also the relative gene expression level was assessed by using $2^{-\Delta\Delta Ct}$ quantitative method (23), in which the parameter threshold cycle (Ct), demonstrates the fractional cycle number where the fluorescent signal reaches detection threshold. The results were normalized to an endogenous control (*GAPDH*). For normalizing each target gene sample, the relative abundance value obtained is divided by the value derived from the endogenous control.

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP-qPCR was carried out using anti-CREM (ab54625, Abcam) and anti-ACT (ab97396, Abcam) antibodies and with the low cell number ChIPkit (Diagenode, Belgium) according to its presented instruction. Input control DNA and immunoprecipitated DNA and were measured by qPCR on a Step One Plus Real-Time PCR System (AB Applied Biosystems, USA) using SYBR Green master mix and designed primers (Table 1) in three groups: OA (n=10), SMA (n=10), SCOS (n=10). qPCR profile was as follows: initial denaturation at 95°C for 4 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 1 minute. The obtained results were normalized to input DNA and presented as percentage of input DNA.

Table 1: Primers designed for this study

Gene	Primer sequence (5´-3´)	Product size (bp)	Annealing temperature (°C)
qPCR primers			
ACT	F: CCATTA*GTGGTCTCACAGGT* R: GTCTCCTAGATGTCAGTGTCC	178	60
CREM	F: ATGACAAATTCAGGAGCTCCTC R: TGGGACAAAGAACTGCTGTG	90	60
PRM1	F: AATAGCACATCCACCAAACTC R: CAACATTTATTGACAGGCGG	134	60
PRM2	F: GCTGGAAGTTAAGAGAAAGTCAC R: GGCTTGAGCATTTGATGTAGG	80	60
TNP1	F: GACCTGATGTTAGATCAAAGCC R: ATTCCTCATTTCGTCACAACTG	75	60
TNP2	F: GGAAATCCAACTAATGAGACCG R: TAGTGTTGCGTAGAAATCACCA	127	60
GAPDH	F: CTCATTTCCTGGTATGACAACGA R: CTTCCTCTTGTGCTCTTGCT	121	60
ChIP-qPCR primers			
PRM1	F: GGAGGAGTCATCTTGTATCG R: TCATTGTGAGGGCAAAGG	147	60
PRM2	F: CTTCCAAATGACAATGTGCG R: TTGCCTTGCCTGTAAAGC	128	60
TNP1	F: GTCTCTTGTACTCATCCAATGCC R: TACTGTGCTGTCACTCACCT	187	60
TNP2	F: TTCTTCTAATGTCCGAATGAGG R: CTGAACAAGTCCCAGTTTCC	76	60

Table 2: Clinical features of patients group

Patient groups	No. of patients	Genetic analysis	Age (Y)	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)
OA	10	46XY/ normal AZF	35.3 ± 2.4	7.7 ± 1.2	8.9 ± 1.3	3.4 ± 0.5
SMA	20	46XY/ normal AZF	31.1 ± 1.4	7.7 ± 1.1	10.3 ± 1.3	4.9 ± 0.4
SCOS	10	46XY/ normal AZF	34.9 ± 1.9	8.8 ± 1.6	20.4 ± 2.3	3.7 ± 0.2
P value	-	-	NS	NS	0.001***	NS

Values are mean ± SEM. NS; Not statistically significant, Kruskal-wallis test, LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, SCOS; Sertoli cell-only syndrome, and ***; P≤0.001.

Statistical analysis

Statistical comparisons between the three groups were performed using Kruskal-Wallis and Spearman's rank correlation was used to determine the possibility of correlation. Differences between groups were considered to be statistically significant at P≤0.05. All statistical analyses were performed by GraphPad Prism (version 8.0.2 for Windows, GraphPad Software, La Jolla California, USA).

Results

SCOS group had the highest serum FSH concentration among all groups

The results demonstrate that the differences in age, LH and testosterone levels among the three groups of OA, SMA and SCOS were non-significant. However, the difference regarding FSH concentration among foregoing

groups was significant (P=0.001, Table 2). Moreover, SCOS group had the highest concentrations of FSH in comparison to OA and SMA (P≤0.005) and there was no significant difference in the FSH concentration between SMA and OA group.

ACT and *CREM* expressions are downregulated in SMA group

ACT, *CREM* and its target genes (*TNP1, TNP2, PRM1* and *PRM2)* expressions were evaluated by qPCR analysis in the three groups. Quantification of mRNA relative expression showed significant lower expression of *ACT* in SMA group in comparison to positive control group (P≤0.05, Fig.1). *CREM* expression level also significantly lowered compared to OA group (P≤0.002, Fig.1). Significant decrease in expression levels of *TNP1, TNP2, PRM1* and *PRM2* in testis tissues of round SMA and SCOS groups were observed in comparison to positive control group (Fig.2). The significance was calculated at (P≤0.05).

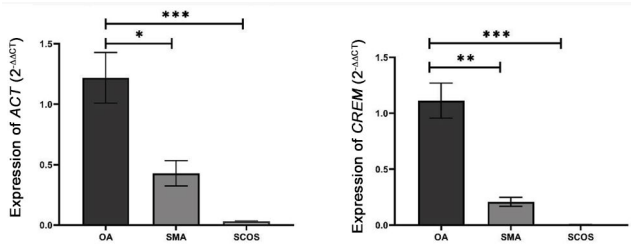

Fig.1: Relative mRNA expression of the *ACT* and *CREM* genes normalized to *GAPDH* in testicular samples with OA (n=10), SMA (n=20) and SCOS (n=10). OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, SCOS; Sertoli cell-only syndrome, ***; P≤0.001, **; P≤0.002, and *; P≤0.05.

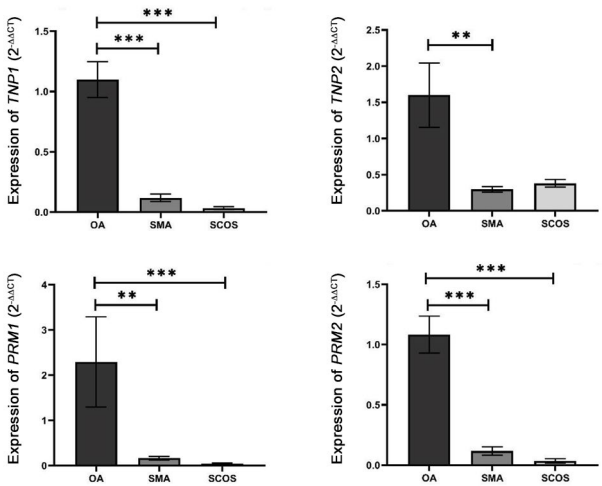

Fig.2: Relative mRNA expression of the *TNP1, TNP2, PRM1* and *PRM2* genes normalized to *GAPDH* in testicular samples with OA (n=10), SMA (n=20) and SCOS (n=10). OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, SCOS; Sertoli cell-only syndrome, ***; P≤0.001, and **; P≤0.002.

TNP1 expression is positively correlated with *PRMs* expressions in both SMA group and OA group

The obtained results from Spearman's correlation coefficient test from SMA group revealed positive correlations between *TNP1* and *PRM1* and between *TNP1* and *PRM2* (P≤0.05, two-tailed). Furthermore, *PRM1* and *PRM2* were positively correlated (P≤0.05, two-tailed). However, no correlation was observed between *TNP2* and *PRMs* genes. In OA group, we only observed positive correlation between *TNP1* expression level and *PRMs* expression levels (P≤0.05, two-tailed). The results are presented in Tables S1 and S2 (See Supplementary Online Information at www.celljournal.org).

ACT and CREM proteins are associated with promoter regions of *TNP* genes

Total protein levels of ACT and CREM into regulatory regions of *PRM1, PRM2, TNP1* and *TNP2* genes in the testis tissue sections were assessed by ChIP real-time PCR. The quantitative data revealed a significant decrease in detection of CREM and ACT transcription factors into regulatory regions of *PRM1, PRM2, TNP1* and *TNP2* in testis tissues of SMA and SCOS groups in comparison to positive control group (Figs.3, 4). The significance was calculated at P≤0.05.

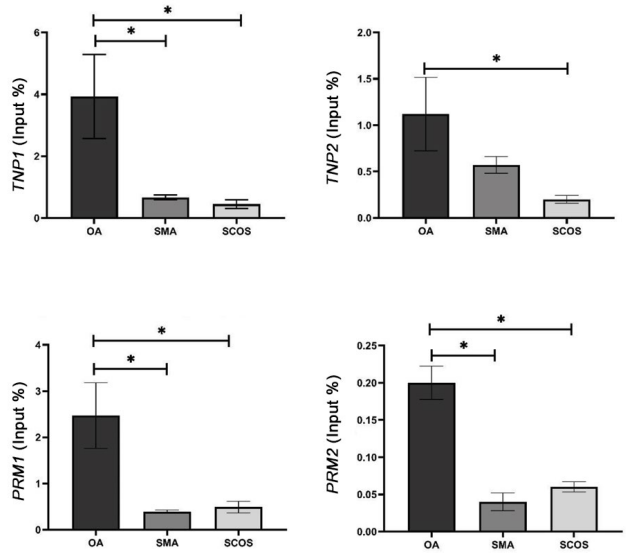

Fig.3: Incorporation of CREM protein on the promoter regions of *TNP1, TNP2, PRM1* and *PRM2* genes in patients with OA (n=10), SMA (n=10) and SCOS (n=10). OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, SCOS; Sertoli cell-only syndrome, and *; P≤ 0.05.

The correlations between incorporations of ACT/CREM and the expressions of *PRMs*/*TNPs* genes

CREM and ACT incorporations on *PRM1* were found to be negatively correlated with *PRM1* expression in SMA group (P≤0.05, two-tailed). Moreover, ACT incorporation on *TNP2* was found to be positively correlated with *TNP2* expression (P≤0.05, two-tailed). Other correlations

between ACT/CREM incorporations and their target genes' expressions were non-significant. For OA group, we only observed negative correlation between ACT incorporation on *TNP2* and *TNP2* expression (P≤0.05, two-tailed). All results are presented in Tables S3 and S4 (See Supplementary Online Information at www.celljournal.org).

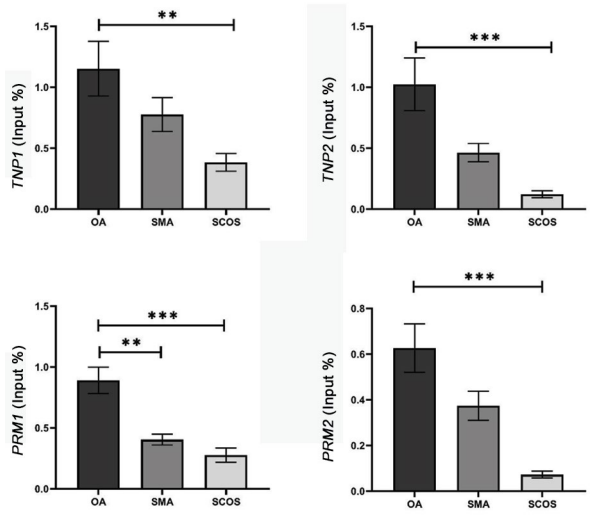

Fig.4: Incorporation of ACT protein on the promoter regions of *TNP1, TNP2, PRM1* and *PRM2* genes in patients with OA (n=10), SMA (n=10) and SCOS (n=10). OA; Obstructive azoospermia, SMA; Round spermatid maturation arrest, SCOS; Sertoli cell-only syndrome, ***; P≤0.001, and **; P≤0.002.

Discussion

Both environmental factors and genetic factors can attribute to male infertility which this fact simply indicates that this condition is perplexing and multifactorial, and thousands of genes are known to be involved in male infertility (24). Therefore, delving into molecular characterization of male infertility can help better understand this medical condition and can also contribute to its treatment.

In the present research, we investigated the expression and chromatin incorporation of ACT and CREM together with the expression of their target genes in the testis of infertile men with SMA. SMA group was compared with both patients classified in OA group and SCOS group. OA group was considered as a positive control group because OA patients undergo normal spermatogenesis, but their reproductive tracks are blocked and SCOS group were assigned as negative control group due to lack of germ cells in this group (5, 25). Our data demonstrate that there is low expression of ACT and CREM in infertilities associated with post-meiotic arrest.

Our findings are consistent with previous studies which have demonstrated that male mice which lack the functional form of *CREM* gene in its genome, are infertile with arrested spermatogenesis at the round spermatid stage (26, 27). ACT regulates CREM expression level in male germ cells and it is also required for spermatid maturation (22, 28). A study showed that male mice which lack *ACT* expression are still fertile but show significantly decreased numbers of mature sperms and severe abnormalities of the remaining cells (22).

CREM and its cofactor ACT cooperate to initiate the expression of several important post-meiotic genes including the expression of *TNP1*, *TNP2*, *PRM1*, *PRM2* which are necessary for chromatin compaction through spermatogenesis (15-17). It has been demonstrated that transgenic mice carrying null mutations for *TNP1* and *TNP2* are infertile associated with decreased chromatin compaction, motility, viability and a high proportion of morphology abnormalities of sperm cells (11). Another study demonstrated that null mutation for *PRM1* and *PRM2* genes in mice, interrupts nuclear formation and the normal function of the sperm ultimately leading to infertility (29).

We observed low incorporations of ACT and CREM on their target genes and low expressions of *TNP*s and *PRM*s. Since post-meiotic cells are absent or less abundant in the samples with SMA samples and these cells normally express these genes, therefore, it is expected that we observe a decrease in incorporation of ACT and CREM and decreased levels of expression of their target genes compared to samples which have all spermatogenesis cell stages including post-meiotic cells.

TNP1 is located on chromosome 2 and it has a vital role in decreasing the melting temperature of DNA and relaxing its nucleosome core particles which are essential for histones eviction. On the other hand, *TNP2* and the *PRMs* are in a cluster on chromosome 16 and they are mainly responsible for DNA compaction (30). We carried out correlation analyses between *TNP*s and *PRM*s gene expressions in SMA group and we observed that *TNP1* expression is positively correlated with both *PRM*s genes. This finding is somewhat surprising since these genes are not neighboring genes, but it suggests that are co-regulated. We also found that *PRM1* was positively correlated with *PRM2*. Same correlation patterns were observed for OA group except that *PRM1* was not correlated with *PRM2*.

We also ran correlation analyses between the incorporation of ACT/CREM and the expression of the *PRM*s/*TNP*s genes in both OA group and SMA group. In OA group we only observed negative correlation between the incorporation of ACT on *TNP1* and *TNP1* expression. Moreover, we found out that incorporations of ACT and CREM on *PRM1* regulatory region are negatively correlated with *PRM1* expression in SMA group which might suggest that ACT and CREM incorporations are increased in order to compensate for the reduced expression of *PRM1* in SMA group.

Conclusion

This work confirms that ACT and CREM have key roles in spermatid maturation and decrease in their expression levels might be associated with spermatogenesis failure at the stage of post-meiotic cells (SMA group). However, further investigation on larger sample size is required to justify these findings.

Acknowledgments

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Authors' Contributions

P.J.; Statistically analyzed the experimental data and drafted the manuscript. R.F.; Supported technical performance and data analysis. M.A.S.G.; Provided expertise, urologist and the administrative supporter for collecting samples. M.Sh.; Provided expertise, designed and supervised the research and took part in revising the manuscript. All authors studied and approved the final manuscript.

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