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Research Article

All Trans Retinoic Acid Adjusts Gene Expression Profiling of Spermatogenesis Pathway in Elastase Treated Rat Lungs

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Abstract

Pulmonary emphysema is a chronic lung disease and is due to persistent inflammation. The inflammatory mediators generated during emphysema are thought to be infiltrated into the circulatory system, leading to systemic inflammation and hence, affecting function of other vital organs, here, a spermatogenesis process in testicular tissue. The impending role of ATRA towards the molecular mechanisms prevailing systemic inflammation due to inflammatory mediator's overspills in emphysema is inadequately implicit.

Therefore, the present study deals with investigating the potential effect of ATRA on physiological functions of testis tissue in an established elastase induced emphysema rat model. Three experimental groups (i.e. control, SS; emphysema, ES; and therapy, EA) were prepared. Subsequently, testis from each rat was collected for tissue-histopathology and real-time PCR based mRNA expression analyses. Independent to this, for understanding the interaction between ATRA with target proteins an in silico study was conducted.

Testis histopathology photomicrographs clearly shows a decrease in number of leydig cells in ES than SS, however, an increase in number of leydig cells was seen in EA. The mRNA expression of NF-κB, TNF-α, TNFR1A, AMH and NANOS2 was up regulated in ES group as compared to SS group while the same were significantly reduced in EA group. However, a significant reduction in the mRNA expressions of the male fertility genes that is FSHR, RBM3, DAZL, CDYL and TGFβ1were obtained in ES group than SS group, nevertheless in ATRA supplemented group (EA), the mRNA levels were increased significantly. Not only this, a reduction of CAT and GPx activities in testis tissue of elastase treated lungs (ES) was noticed than the SS group and an induction in the levels of CAT and GPx was obtained in EA group as compared to ES. In conclusion, ATRA supplementation has proven to be beneficial in reducing the inflammation along with maintaining the normal testis architecture as well as the male fertility genes.

Keywords: Chronic Obstructive Pulmonary Disease (COPD); Retinoic Acid; Spermatogenesis Pathway; Elastase Treated Rat Lungs

Introduction

Chronic obstructive pulmonary disease (COPD) is a broad category of lung disorder. COPD is characterized by chronic airflow limitation resulting from excessive airway inflammatory response mediated by cigarette smoke. The disease affects millions of Americans and is the fourth leading cause of disease-related death in the U.S. [1]. There are two major complications which come under

COPD, the first one is the chronic bronchitis and the second one is pulmonary emphysema. Chronic bronchitis refers to the overproduction of cough or mucus via goblet cells while the pulmonary emphysema characterized by shortness of breathing due to the damaged alveolar septa and lung tissue destruction. These conditions caused by long term exposure of harmful gases and mainly by cigarette smoking; these things can develop pulmonary inflammation and destruct the alveoli of the lung directly and is thought to be irreversible.

Recently, the concept of systemic inflammation as a consequence of spillover of inflammatory mediators from the lungs to the systemic compartment in COPD has been broadly discussed [2]. It has been hypothesized that inflammation in the lung may linked by impaired lung function and results from lung to plasma spillover of inflammatory mediators, such spillage of inflammatory mediators may further be affects the proximal as well as distal organs and their important associated molecular signaling pathways [3].

Spermatogenesis is an indispensable process for the continuity of life and requires germ cells production. Sperm development demands a series of substantial molecular and morphological changes in male germ cells. In mammals, spermatogenesis process takes place inside seminiferous tubules of testis. The requirement for frequent production of large number of spermatozoa supports the various necessities on spermatogenesis. The spermatogenesis process involves proliferation of spermatogonia along with two meiotic divisions followed by spermiogenesis, spermiation and sperm maturation stages to develop sperms. Differentiation of spermatogonia, spermatocytes, and spermatids via noticeable advancement of mitotic expansions, meiotic reduction divisions, and morphological transformations needs various growth factors and hormones to regulate the critical steps resulting in the accomplishment of the generation of spermatozoa [4]. It has been reported that there are some of the genes, which are involved in regulating the spermatogenesis process [5]. However, when the levels of cytokines such as tumor necrosis factor-alpha (TNF–α), interleukin–1 alpha (IL–1α) and interleukin 1 beta (IL–1β) are higher than normal, as seen in conditions of inflammation, they become very harmful to sperm production [6]. Moreover, inflammation is also associated with oxidative stress and the latter is well known to impair sperm function and resulted in generation of infertility in males [5].

All-trans-retinoic acid (ATRA) has been manifested to counter the emphysema initiated through elastase in experimental rat model. ATRA is an active metabolite of vitamin A under the family retinoid [7]. ATRA has been reported to possess an anti-inflammatory features [8]. The realization that ATRA improves structural and functional lung destructions in an elastase-induced model of emphysema provoked immense curiosity within the scientific fraternity. An exogenous supplementation of ATRA can encourage alveolar regeneration in emphysematous mouse and rat model with damaged alveolar development by reducing ongoing inflammation. Alveolar regeneration with ATRA may therefore be an essential therapeutic strategy for the remedy of pulmonary emphysema. However, the present study was designed to determine the following questions (Figure 1):

1) To examine the effect of inflammatory mediator's spillover due to elastase treatment from the lung to the testicular tissue, particularly does it is affecting spermatogenesis process?
2) To examine the prospective role of ATRA in rectification of spermatogenesis process of testicular tissues of elastase treated lungs.

Materials and Methods

***Insilico* study analysis:** AutoDock is widely used tool for understanding the interaction between ligand and target proteins. Autodock 4.2.6 was employed for building interactions of ATRA to all target proteins (TNF-α, TNFR1A, NF-κB, AMH, DAZL, CDYL, TGFβ1 and NANOS2). For performing autodocking, Autodock 4.2.6 tool was downloaded along with Discovery Studio Visualizer 2.5. ATRA ligand 3D structures along with structures of target proteins were downloaded in PDB format from PubChem, PDB (Protein Data Bank) and SWISS-GROUP. AutoDock 4 was executed. Obtained docked poses were played in different conformations to observe hydrogen bonds and interaction energies and best-docked poses of interaction of ATRA to all target proteins were selected. The interactions were detected using Discovery Studio Visualizer 2.5.

Elastase and ATRA treated experimental rat model: All experimental models were prepared according to the procedure and dosing described by [9] and were in compliance with National and International guidelines approved by the regional government (IAEC, Ministry of Environment and Forests, India. INM/IAEC/2012/05).

Figure 1: Hypothesis of the current study regarding effects of inflammatory mediator's spillover from emphysematous lungs on testis tissue and prospective role of ATRA in minimizing such spillage of inflammatory mediators. First objective is to investigate the effect of porcine pancreatic elastase supplementation in the lung of rat causes chronic inflammation, leading to emphysematous changes. Such chronic inflammation releases Inflammatory mediator's "spill over" into the systemic circulation and infiltrates into the testis tissue. The spillover of inflammatory mediators may also induce dysregulation of physiological processes and leading its influence in spermatogenesis process in the testicular tissue. The second objective is to examine the prospective role of ATRA in minimizing the effect of inflammatory mediators on spermatogenesis process in the testicular tissue.

The models were prepared at Institute of Nuclear Medicine and Allied Sciences, DRDO, New Delhi under the supervision of Dr. Amit Kumar Tyagi. Pathogen-free eight weeks old male, sprague dawley rats (approximately 150g body weight) were randomly assigned to three different experimental models (n = 4 per group). Rats were maintained under anesthesia by isoflurane and were given either elastase/saline oropharyngeally or ATRA/olive oil intraperitoneally. Animals were sacrificed on day 38 by cervical dislocation. Blood was removed by performing ventilation/perfusion with sterile PBS. The vital organs like testis was harvested from the body and was dipped immediately in liquid nitrogen tank and were stored at -80°C until analyzed for investigation of relative mRNA expression while the left testis was kept for histopathological analysis purpose.

RNA isolation and RNA purity measurement: To determine the relative mRNA expression in testis tissue, total RNA was isolated using RNeasy Mini Kit (Qiagen, New Delhi, India) based technique and according to the protocol by [9]. The quantity and purity of total RNA was determined with Nanodrop spectrophotometer (Thermo Scientific, New Delhi, India). The obtained ratio of 260 nm/280 nm was > 1.8 but < 2.1 for all the samples.

cDNA synthesis and relative mRNA expression: While cDNA was synthesized by introducing equal amounts of RNA (200 ng) from each sample in a total reaction volume of 20 µl using an Oligo dT primer (Qiagen, New Delhi, India) and Omniscript RT Kit (Qiagen, New Delhi, India) and their respective protocol. The reaction was incubated at 37°C for 1h in Thermoblock TB2 (Biometra, New Delhi, India). The quantitative real time PCR for determining the amplification factor of the target genes (Table 1) were performed using real-time PCR (Agilent technologies). QuantiTect SYBR Green PCR Kit (Qiagen, New Delhi, India) was used for PCR and the standard protocol was followed. The thermal cycle conditions used for all reactions were as follows: Step 1: 95°C for 15 min; a 30 cycles of Step 2 (95°C for 40 sec), Step 3 (sequence-specific oligonucleotide primer's annealing temperature for 30 sec) and Step 4 (72°C for 40

sec), followed by one time of step 5: 72°C, 5 min. The expressions of test genes were normalized by using endogenous control that is GAPDH according to the formula 2 to the power of delta cycle threshold ($2^{\Delta Ct}$), where ΔCt = Ct, reference gene – Ct, test gene.

Sl. No.	Gene name	Left Primer (5'-3')	Right Primer (5'-3')
1	*Rattus norvegicus* follicle stimulating hormone receptor (Fshr), mRNA	CGCTCATCACTGTGTCCAAG	AAGTTGTGGGTAGCGGATGA
2	*Rattus norvegicus* anti-Mullerian hormone (Amh), mRNA	CTGTTTGGCTCTGATTCCCG	GTCTCTAGGAAGGGGTCAGC
3	*Rattus norvegicus* RNA binding motif protein 3 (Rbm3), mRNA	CTCTGTTCTCCCGGTTCCTT	CTTTGAGTCTCCCGGTCCTT
4	*Rattus norvegicus* deleted in azoospermia-like (Dazl), mRNA	CATACATGCAGCCTCCAACC	CATTGGGCAAAAGGTCAGCT
5	*Rattus norvegicus* chromodomain Y-like (Cdyl), mRNA	TAGCGTCCCACTTGTTCCTT	TGCTTTGCCTTGAATGCCAT
6	*Rattus norvegicus* transforming growth factor, beta 1 (Tgfb1), mRNA	TCGCTTTGTACAACAGCACC	ACTGCTTCCCGAATGTCTGA
7	*Rattus norvegicus* nanos C2HC-type zinc finger 2 (Nanos2), mRNA	GGAGGATGAGGTGTCTGAGG	GACACACTACCACCCCTTCA
8	*Rattus norvegicus* nuclear factor kappa B subunit 1 (Nfkb1), mRNA	TTCCTGCTTACGGTGGGATT	CCCCACATCCTCTTCCTTGT
9	*Rattus norvegicus* hypoxia inducible factor 1 subunit alpha (Hif1a), mRNA	CCAGCAGACCCAGTTACAGA	TTCCTGCTCTGTCTGGTGAG
10	*Rattus norvegicus* signal transducer and activator of transcription 3 (Stat3), mRNA	TCAGTGAGAGCAGCAAGGAA	TTTCCGAATGCCTCCTCCTT
11	*Rattus norvegicus* interleukin 1 beta (Il1b), mRNA	GGGATGATGACGACCTGCTA	TGTCGTTGCTTGTCTCTCCT
12	*Rattus norvegicus* interleukin 6 (Il6), mRNA	CTCATTCTGTCTCGAGCCCA	CTGTGAAGTCTCCTCTCCGG
13	*Rattus norvegicus* tumor necrosis factor (Tnf), mRNA	ACACACGAGACGCTGAAGTA	GGAACAGTCTGGGAAGCTCT
14	*Rattus norvegicus* lymphotoxin alpha (Lta), mRNA	TCCTGCCTCTTCTCTTGAGC	CCATGGGTCAAGTGCTTCTG
15	*Rattus norvegicus* interferon gamma (Ifng), mRNA	CGTCTTGGTTTTGCAGCTCT	CGTCCTTTTGCCAGTTCCTC
16	*Rattus norvegicus* tumor necrosis factor (Tnf), mRNA	ACACACGAGACGCTGAAGTA	GGAACAGTCTGGGAAGCTCT
17	*Rattus norvegicus* TNF receptor superfamily member 1A (Tnfrsf1a), mRNA	ATTCACCAGCGTTGCCAATT	TATCCCTCTTCTCCCGGTCA
18	*Rattus norvegicus* caspase 8 (Casp8), mRNA	GGTTACAGCTCTCCTACCCC	TGTCTTCCTCCAACATCCCC
19	*Rattus norvegicus* caspase 3 (Casp3), mRNA	CATGCACATCCTCACTCGTG	CCCACTCCCAGTCATTCCTT
20	*Rattus norvegicus* cytochrome c, somatic (Cycs), mRNA	GGCAAGCATAAGACTGGACC	GTCTGCCCTTTCTCCCTTCT
21	*Rattus norvegicus* caspase 9 (Casp9), mRNA	AACAACGTGAACTTCTGCCC	CCATCTCCATCAAAGCCGTG
22	*Rattus norvegicus* tumor protein p53 (Tp53), mRNA	CTCCTCTCCCCAGCAAAAGA	GTAGACTGGCCCTTCTTGGT

Table 1: List of primers of *Rattus norvegicus* for mRNA expression.

Histopathology of testis tissues: Right testis tissues were fixed in 6% phosphate-buffered paraformaldehyde and stored in the refrigerator (4°C). Tissue blocks were prepared using molten paraffin and allowed to cool and solidify before making tissue sections. 10 µm thin tissue sections were cut using a microtome (Spencers rotary microtome, India). Subsequently, tissue sections were deparaffinised three times by Xylene, rehydrated with different concen-

trations of ethanol and stained with hematoxylin and eosin (H&E) stain. The stained tissues were further subjected under the microscope to assess the changes in the structure of tissues within the different animal models.

Assessment of antioxidant status: CAT and GPx are some important antioxidants that protect the tissues from oxidative damage. CAT assay was performed by incubating 0.1 ml of tissue homogenates in 50 mM hydrogen peroxide (30%) in 0.0059 mM potassium phosphate (pH 7) and was placed in a cuvette in a spectrophotometer which was adjusted to 240 nm and 25°C. The decrease in absorbance at 240 nm for 2 - 3 minutes was finally recorded. CAT activity of enzyme was expressed as units/mg protein:

Units/mg = (ΔA240/min) x 1000/43.6 x mg enzyme/ml reaction mixture.

For the estimation of GPx in testis tissue samples of different groups, 0.1 ml of tissue homogenate was mixed with 0.1ml EDTA (1 mM), 0.1ml sodium azide (1 mM), 1.44 ml phosphate buffer (0.1M, pH 7.4), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2 mM) and 0.01ml H_2O_2 (0.25 mM). At 340 nm and 25°C, the depletion of NADPH was recorded. The enzyme activity was calculated as µmol NADPH oxidized/minute/mg protein with the molar extinction coefficient of 6.22 X 103 M^{-1} cm^{-1}:

GPx = ΔO.D x Vol of assay x 1000/6.22 x Vol. of enzyme x protein mg.

Statistical analysis: Unless stated otherwise, mean values ± s.d. are given. Student's unpaired t-test was used to determine the level of significance of differences between Control versus Elastase treatment and Elastase treatment versus ATRA treatment, respectively. All analyses were performed by means of GraphPad Prism version 5, San Diego, USA. Levels of significance are indicated by * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Results

Effect on the binding of target genes with the ATRA: Prior to testing the proposed hypothesis, the potential effect of ATRA has been checked by using in silico approach. The comparative optimization was carried out to find the best-docked pose and the energy of all target proteins (TNF-α, TNFR1, NF-κB, FSHR, AMH, DAZL, CDYL, TGFβ1 and NANOS2) with ATRA. The best 10 docked poses were identified and analyzed. All poses were analysed along with their binding energies. From among ten, one best-docked pose and its binding energy was selected of all target proteins with ATRA. The interaction of all target proteins with ATRA was generated and visualized through Discovery Studio Visualizer 2.5. Figure 2 displays the best-docked pose of ligand ATRA to target proteins FSHR, AMH, DAZL, CDYL, TGFβ1 and NANOS2 acquired with binding energy or ΔG of -6.84, -5.75, -5.72, -6.01, -5.79 and -7.13 kcal/mol respectively and inhibition constant (Ki) of 9.67 µM, 61.37 µM, 28.23 µM, 64.53 µM, 39.16 µM, 57.33 µM and 5.97 µM respectively.

Effect of elastase and ATRA on antioxidant level: Using tissue extract of testicular tissue, we have examined the effect of elastase

Sl. No.	Receptor	Ligand	Binding Energy (kcal/mol)	Inhibition constant (Ki)	Ligand efficiency	Hydrogen bonds formed
1.	FSHR	ATRA	-6.84	9.67µM	-0.31	Protein C: ILE111 Protein C: LYS133
2.	AMH	ATRA	-5.75	61.37µM	-0.26	Protein B: ARG495 Protein B: ARG553
3.	DAZL	ATRA	-5.72	64.53µM	-0.29	Protein A: GLN100 Protein A: LYS107
4.	CDYL	ATRA	-6.01	39.16µM	-0.27	Protein B: ASN207
5.	TGFβ1	ATRA	-5.79	57.33µM	-0.26	Protein C: GLN349
6.	NANOS2	ATRA	-7.13	5.97µM	-0.32	Protein A: LYS80 Protein A: HIS107

Table 2: Interaction of ATRA with target proteins.

Figure 2: Interaction of ATRA to target proteins involved in spermatogenesis pathway using in silico analysis. The image depicts the ATRA ligand binding to the amino acids of target proteins. ATRA ligand is displayed in green colour. Hydrogen bonds formed between ATRA ligand and amino acids of target proteins are displayed as green dotted line. A: Interaction of ATRA to FSHR protein. B: Interaction of ATRA to AMH protein. C: Interaction of ATRA to RBM3 protein. D: Interaction of ATRA to DAZL protein. E: Interaction of ATRA to CDYL protein. F: Interaction of ATRA to TGFβ1 protein. G: Interaction of ATRA to NANOS2 protein.

and ATRA on antioxidant level. The antioxidant level was determined by studying CAT and GPx activities. Here, the CAT and GPx activities were found to be considerably reduced in testis tissue of elastase treated lung group (ES) as compared to the healthy ones (SS). Interestingly the favorable role of ATRA has been noticed in testicular tissue of elastase treated and ATRA received lungs (EA) than the testicular tissue of elastase treated lung, where the levels of CAT and GPx were significantly up-regulated and the activities of CAT and GPx were restored.

Effect of elastase and ATRA on gene expression profiling of key genes for spermatogenesis: Using testicular tissue homogenate, the mRNA levels of marker genes for spermatogenesis pathway (FSHR, AMH, RBM3, DAZL, CDYL, TGFβ1 and NANOS2) and pro-inflammatory cytokines (NF-κB, TNF-α and TNFR1A) were studied. As shown in figure 4A-4C, the mRNA expression of pro-inflammatory genes (TNF-α, TNFR1 and NF-κB) was significantly up-regulated in testicular tissue of elastase treated lungs group (ES) as compared to control group (SS) while they were consider-

Figure 3: CAT and GPx assays: The graphs depict in reduction of CAT and GPx activities in testis tissue of elastase treated lungs (ES) than the testis of saline treated lungs. Nevertheless, a significant induction in the levels of CAT and GPx was noticed in testis of ATRA supplemented elastase treated lungs as compared to ES. Graph represents mean values with standard deviation. Unpaired t-test was performed to analyse effect of elastase and ATRA. *p < 0.05.

ably down-regulated in ATRA supplemented elastase treated group (EA) and were identical to the control group (SS). Similar trend in mRNA expression of Anti-mullerian hormone (AMH) and NANOS2, spermatogenesis pathways genes, were also recorded (Figure 4D and 4E). However, the mRNA levels of FSHR, DAZL, CDYL and TGFβ1 were markedly decreased in testis tissue of elastase treated lungs (ES group) than the healthy ones (SS group). Here too, ATRA has shown its prospective signature in inducing up their mRNA levels (EA) than ES group (Figure 4F-4I).

Effects of elastase and ATRA on testicular tissue architecture: Histopathological based analysis clearly indicated that the number of leydig cells were decreased in the testis tissue of elastase treated lung (ES) group than healthy ones (SS group). However, in the testicular tissue of ATRA supplemented elastase treated lungs (EA group), there is an increased in the number of leydig cells and was comparable to the control group (SS) as shown in figure 5.

Discussion

The present study reveals the impending effect of ATRA supplementation on the testicular tissue of elastase induced emphysematous rats. It is apparent from previous studies that emphysema involves spillage of inflammatory mediators [3,10]. Here, we assume that such spillage of inflammatory mediators and their infiltration from lung into the testis tissue might have occurred due to systemic inflammation caused by oropharyngeally instilled porcine pancreatic elastase into the lungs of rat in an emphysematous model. If this is the case, then, firstly, a question was addressed here, that is, does spillover of inflammatory mediators from the lung to the testis tissue is having any impact in the dysregulation of spermatogenesis and physiological processes? Secondly, does ATRA which is having an anti-inflammatory property have a potential towards attenuation of such dysregulation of spermatogenesis mechanism due to spillage of inflammatory mediators? To validate above mentioned hypothesis, mRNA expression profiling of inflammatory mediator genes and marker genes for spermatogenesis process were studied, antioxidant levels were measured, and histopathology of testis tissue was examined. Independent to this, in silico study was also been done for determining the binding ability of ATRA with the target proteins.

Our *in silico* results (Figure 2), provided us the foremost clue regarding the probable binding of ATRA with target proteins that are necessary for triggering signaling mechanisms of spermatogenesis pathway. Results obtained signified that ATRA binds to all the target proteins of spermatogenesis pathway. To determine the best docked pose among other conformations, interactions must have lowest binding energy and inhibition constant along with maximum number of H-bond formation between the ligand and protein. ATRA exhibit more binding efficiency to AMH and TGFβ1compared to other target proteins on the basis of autodocking results (Table 1) where their ligand efficiency is -0.26, highest to all values obtained from interactions of ATRA to other proteins. The binding of ATRA to target proteins confirms that potential interactions between them are possible.

During chronic inflammation phase, some immune cells such as leukocytes and neutrophils gets activated and release reactive oxygen species (ROS) which ultimately leads to oxidative stress, which is one of the major cause for tissue injury [11]. While on the other hand, anti-oxidants (such as CAT and GPx) are those that protect the tissues from such oxidative damage. Increased oxidative stress, together with reduced antioxidant defense are involved in the pathophysiology of α-1 antitrypsin [12]. Similarly, some find-

Figure 4: Relative mRNA expression of inflammatory marker and marker genes for spermatogenesis pathway. The mRNA expression of NF-κB, TNF-α, TNFR1A, AMH and NANOS2 was up regulated in ES group as compared to SS group. However, upon ATRA supplementation to elastase treated lungs of testis tissue (EA group), the mRNA expression of the same were significantly reduced than ES group. While the remaining marker genes of spermatogenesis pathway i.e. FSHR, RBM3, DAZL, CDYL and TGFβ1 has shown reduction in mRNA expression in elastase treated group (ES) than healthy group (SS), nevertheless in ATRA supplemented group (EA), the mRNA levels were increased significantly (Figure 4F-4J) than ES group. Data were analyzed by means of unpaired t-test to test for the effect of ATRA and elastase, respectively. *$p < 0.05$; **$p < 0.01$.

ings also suggest that CAT activity has been found to be reduced in COPD patients/animal model for emphysema [8]. Here, to assess the oxidative stress's response in the testis tissue, we have performed biochemical assays for determining the levels of GPx and CAT activity. Our results suggested with the use of testis tissue isolated from elastase treated lung group (ES) is showing significant decrease in the endogenous CAT and GPx activities when compared to the control group. Reduced GPx activity provides us a clear cut hint towards its involvement in tissue damage as reduced level of anti-oxidant is in direct proportion to the severity of tissue damage. However, the most important finding is here regarding what happen after ATRA supplementation to the activities of GPx and CAT? Interestingly, an increase in the levels of CAT and GPx were recorded in testis tissues received ATRA in elastase treated lungs as compared to the testis isolated from emphysematous group. The possible reason behind improvement in the activities of CAT and GPx is might be due to the tissue regenerative property of ATRA supplementation that further involves in the restoration of CAT and GPx activities in the testis tissue of emphysematous condition.

To confirm the spillage of inflammatory mediators from lungs to the testis in elastase treated group, we evaluated the mRNA expression of pro-inflammatory cytokines and subsequently consequence of its spillage in the spermatogenesis process. Tumor necrosis factor is a cell signaling protein involved in systemic inflammation and is one of the cytokines that make up the acute phase reaction

Figure 5: Histopathology of testicular tissue. Hematoxylin and Eosin staining tissue sections of testis tissue show normal histology in SS group (A) while ES group (B) showing less number of Leydig cells with abnormal histology and ATRA supplemented group (EA) showing more number of leydig cells (C) with improved histology sections.

by binding to a specific receptor (TNFR1) and involves in the up-regulation of inflammatory reactions. The production of cytokines and its survival is strictly under the control of transcription factor which is called as NF-κB. Our mRNA expression results of TNF-α, TNFR1 and NF-κB has shown a clear cut signature of inflammatory mediator's spillage from elastase treated lungs to the testis organ as the mRNA expression of these genes were increased markedly than the control group. This confirms that inflammatory mediators released due to elastase treatment in the lungs have been spilled over to the testis tissue as well. However, the appealing part of this study regarding mRNA expression of TNF-α, TNFR1A and NF-κB was observed upon ATRA supplementation to the testis of elastase treated lungs. The highly induced mRNA expressions of TNF-α, TNFR1A and NF-κB in testis tissue of elastase group have been reduced significantly upon the supplementation of ATRA (EA group). This indicates that ATRA has shown its positive signature in bringing down the induced expression of inflammatory mediators due to fact that ATRA possesses a strong anti-inflammatory property [13] and also has been formerly demonstrated towards reduction of the TNF-α, TNFR1A and NF-κB mRNA levels in lung tissue of elastase induced emphysema rats [14].

We have picked up some marker genes of male infertility and spermatogenesis process and likewise as stated above, a remarkable up regulation in the expression of these marker genes i.e. AMH and NANOS2 in testis tissue of elastase treated lungs (ES) have been noted. AMH is secreted by immature sertoli cells and is associated to the testicular development and function. A study was conducted to understand the role of AMH in the regulation of Sertoli cells in mice. They found that AMH has proved to reinforce apoptosis process because of the rise of mRNA and protein expression of apoptotic gene [15]. Here, we found similar results and noticed up regulation of AMH expression in testis of elastase treated lungs thus stating that high level of AMH might have an influence in the deregulation of spermatogenesis pathway. However, NANOS2 has been reported to be expressed in many stages of spermatogenesis pathway [16]. NANOS2 plays other roles in the sexual differentiation of male germ cells. In an experiment on the function of NANOS2, it was found that over- expression of NANOS2 in testis of mice has a tendency to reduce proliferation of spermatogonial stem cells along with their differentiation [17]. In the current study, NANOS2 up regulation was observed in testis of elastase treated lungs and this might be affecting spermatogenesis pathway. Nevertheless, ATRA has shown the prospective signature on the expression pattern of these genes, which were induced in ES than SS, by reducing in their expression pattern and brought them close to the control.

Based on the immunohistochemical studies of human testis, it has been noticed that DAZL is expressed in spermatogonia to meiotic spermatocytes [18]. Mutations in DAZL gene have been linked to severe spermatogenic failure and infertility in males, whereas, CDYL belongs to a multigene family called the CDY-related gene family, members of which have been shown to play important roles in mammalian male fertility and spermatogenesis [19]. Nevertheless, transforming growth factor-beta1 (TGF-β1) which is apolypeptide member of the transforming growth factor beta superfamily of cy-

tokines and is a secreted protein that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation, and apoptosis. TGF-β1 is a multifunctional set of peptides that controls proliferation, differentiation, and other functions in many cell types. The well known ligands of TGF-β superfamily, which includes the prototypical TGF-β, has been reported as one of the key regulators of testis development and spermatogenesis. Interestingly here, the down regulation in the expression of these genes were noticed and provided us a clear cut signature of elastase on the spillage of inflammatory proteins from lung tissue to the testis. A remarkable up regulation of these genes expression in testis tissue had further suggested us a potential role of ATRA supplementation, which have an anti-inflammatory effect, was not only limited to the lung tissue but also effective to the testis tissue.

Conclusion

In conclusion, ATRA supplementation has proven to be beneficial in reducing the inflammation along with maintaining the normal testis architecture as well as the male fertility genes.

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