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ORIGINAL RESEARCH

Improvement of sperm function, chromatin damage, and oxidative damage by N-Acetyl cysteine in varicocelized rats model

Marziyeh Tavalaee[1], Maurizio Dattilo[2]* Parisa Mohammadi[1], Mohammad Hossein Nasr-Esfahani[1]†

1. Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran.

2. R&D Department, Parthenogen, Piazza Indipendenza 11, Lugano 6900, Switzerland.

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Abstract: **Introduction:** N-Acetylcysteine (NAC), an acetylated form of the amino acid cysteine and precursor of reduced glutathione, plays important roles in a multitude of cellular processes, such as oxidative damage and detoxification of many electrophiles. Considering the pathophysiology of oxidative stress induced infertility in varicocele, we aimed to investigate the effect of NAC on semen analysis parameters (light microscopy), chromatin structure (aniline blue and acridine orange staining), and lipid peroxidation (BODIPY probe) in varicocelized rats. **Methods:** In this experimental study, varicocelizing surgery was carried out on 30 Wistar rats. Ten of them were sacrificed after two months (one round spermatogenesis), together with control rats (n=10) and sham operated rats (n=10), to verify the varicocele model. Out of the remaining twenty varicocelized rats, ten received NAC while ten were treated with water (control group) for two months. **Results:** All the investigational parameters (sperm parameters, chromatin integrity, and lipid peroxidation) severely worsened 2 and 4 months after surgical varicocele. The administration of NAC for two months significantly improved all the investigational parameters as compared to control rats at four months ($p<0.05$). **Conclusion:** The supplementation of varicocelized rats with NAC was effective in antagonizing the damage as well as in preserving testicular structure and spermatogenetic function. These effects are likely to occur also in clinical varicocele.

Keywords: N-acetylcysteine; Sperm parameters; Chromatin structure; Lipid peroxidation; Varicocele

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1. Introduction

N-acetylcysteine (NAC) is derived from acid L-cysteine, a semi-essential amino acid. L-cysteine is not soluble, but the N-acetylation makes it soluble and bioavailable, thus suitable as a treatment for human and animal use. The main clinical use of NAC is as a substitute of cysteine to feed the endogenous synthesis glutathione (GSH), a main physiologic antioxidant. Besides acting as a precursor of GSH, NAC has its own direct antioxidant activity and both molecules are able to preserve cellular oxidative homeostasis by removing reactive oxygen and nitrogen species (1, 2). A growing body of evidences indicates that GSH deficiency contributes to oxidative stress in different diseases such as Parkinson, HIV, cancer, diabetes, and metabolic syndrome (3, 4). Lack of GSH associates with increased oxidative stress, accumulation of toxins and heavy metals, decreased detoxification ability, instability of cell membrane, failure to repair DNA, cell mutations, and eventually, cell death (5). Several studies have shown that pharmacological doses of NAC can increase intracellular concentration of cysteine/GSH. This can scavenge oxidants and thereby, decrease apoptosis and restore cellular antioxidant capacity (6).

It is well known that the level of oxidative stress is higher in infertile men compared to fertile individuals, which has detrimental effects on sperm function. Ciftci et al. (2009) showed that NAC was able to reduce peroxides and oxidative

*Corresponding Author: Maurizio Dattilo, R&D Department, Parthenogen, Piazza Indipendenza 11, Lugano 6900, Switzerland. E-mail: maurizio.dattilo@parthenogen.ch

†Corresponding Author: Mohammad Hossein Nasr-Esfahani, Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran. E-mails: mh.nasr-esfahani@royaninstitute.org, Phone: 031-95015682

stress index while improving semen volume, sperm motility, and total antioxidant capacity, in idiopathic male infertility (7). Abnormal dilatation of the pampiniform venous plexus and of the spermatic vein in the scrotum is called "varicocele" and is the most common reversible cause of infertility in males. It leads to sperm dysfunction and is detected in almost 40% of infertile men (8). Oxidative stress is known as a key element in the pathophysiology of varicocele and pivotal mediator of testicular damage. Due to scrotal hyperthermia, excess reactive oxygen and nitrogen species, such as hydrogen peroxide and nitrous oxide, are produced by plasma membrane, cytoplasm, mitochondria, and peroxisomes. Excessive production of ROS could lead to low quality in produced sperm, fragmentation of sperm DNA, abnormal chromatin packaging, and other sperm physiological characteristics. In addition, there is significant correlation between oxidative stress and grade of varicocele (9, 10).

A recent review paper (10) concluded that surgery alone is not effective in restoring fertility in men with clinically palpable varicocele and low quality of semen. It is, however, possible that adjuvant therapy with antioxidants provides additional advantages compared to surgery alone (11). Therefore, we aimed to assess the effects of NAC on sperm function, chromatin damage, and oxidative damage, in varicocelized rats as a surrogate model of non-surgically corrected clinical varicocele.

2. Methods

2.1. Animals

Seventy adult Wistar male rats aged 8 to 10 weeks, weight 200 to 250 g, were obtained from Royan Institute for Biotechnology (Isfahan, Iran) and kept under standard laboratory conditions. After a one-week acclimation, the rats were kept under a 12-hour light and a 12-hour dark cycle in room temperature (25±2°C) and were given ad libitum access to food and water. The study was approved by Royan Institute Ethics Committee, and all the experiments were performed in accordance with the relevant guidelines for safe working with animals.

2.2. Experimental design

At zero time of study, 70 rats were divided into seven groups. Varicocelizing surgery was performed according to Ko KW et al (2010) for 40 male rats (12). In brief, after anesthesia with 40 mg/kg, i.p of ketamine (5% ketamine) and 5 mg/kg, i.p. xylazine (2% xylazine), a left renal vein ligation was performed medial to the junction of the adrenal and spermatic veins. Then, anastomotic branches were ligated between the left testicular and left common iliac vein. 20 rats were assigned to the sham group which only has a simple laparotomy with no ligation performed. While for remaining 20 rats, considered as the control group, no surgery or laparotomy was performed. After two months from varicocele induction, 10 rats from each main group (varicocele, sham, and control) were scarified to verify our varicocele model by the assessment of sperm parameters and the use of sperm functional tests. Out of the remaining 20 rats in varicocele groups, 10 received N-acetylcysteine, in which 1/5 tablet of NAC (200mg; HEXAL; Germany) was dissolved in 3ml of water and 500μl of solution was gavaged on a daily basis for two months. The dose of NAC was proportional to the human dose and was calculated based on the formula for conversion from human to animal (13). This was while the remaining 10 varicocele rats were gavaged with water for two months. These rats and the 20 remaining rats from the control (n=10) and the sham (n=10) groups were sacrificed at four months, and sperm parameters and sperm functional tests were assessed (Figure 1).

2.3. Sperm collection

The left side testicles and epididymis of all the study groups were dissected. After removal of unneeded and sidelong tissue, morphometric parameters such as length, width, thickness, weight, and volume, of testis were measured. Caudal sections of all left epididymis were minced and incubated in 5 ml of sperm washing media at 37°C for 30 min before assessing sperm parameters.

2.4. Assessment of sperm parameters

After preparation of sperm suspensions, 20 μl of sperm was introduced into a counting chamber under light microscopy, to assess sperm concentration and motility. Sperm morphology was evaluated by eosin/nigrosin staining. In brief, sperm suspensions were washed in phosphate-buffered saline (PBS) and 20 μl of washed sperm were mixed with 40 μl of eosin for 5 min. Subsequently, 60 μl of nigrosin was added to this mixture, and smears were prepared. For each sample, 200 sperm were counted under a light microscope, and the percentage of abnormalities in the head, neck, and tail regions were reported (14).

2.5. Assessment of sperm chromatin condensation

Sperm chromatin condensation was assessed by aniline blue staining. Sperm washed with PBS were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 120 minutes, and then smears were prepared. After preparation of aniline blue solution, [5% aniline blue was mixed with 4% acetic acid (pH 3.5)], the slides were stained for 90 minutes. For each sample, 200 sperm were counted under a light microscope and percentage of sperm heads with high intensity of aniline blue stain was considered as abnormal chromatin condensation (14).

2.6. Assessment of sperm DNA damage

Sperm DNA damage was assessed by acridine orange (AO) staining. In brief, a drop of washed sperm in PBS was smeared on a slide and fixed with Carnoy's solution (methanol/acetic acid, 3:1) for 2 hours at -4°C. Next, slides were washed with PBS, and stained with acridine orange for 90 minutes. Finally, the slides were washed again with PBS, and 200 sperm were counted for each sample using a fluorescent microscope (Olympus: BX51, Tokyo, Japan). AO incorporates with intact double-stranded DNA to emit green fluorescence, while red fluorescence is related to denatured DNA (14).

2.7. Assessment of lipid peroxidation

Sperm lipid peroxidation was assessed using the fluorescent lipid probe BODIPY 581/591-C11 (D3861, Molecular Probes) according to Aitken et al. (2007) (15). In brief, a final concentration of 5 mM BODIPY C11 was added to 2×10^6 sperm, and incubated for 30 min at 37°C. Then, samples were washed twice with PBS buffer at 650g for 5 min and assessed using a FACSCalibur flow cytometer. Positive controls were obtained after the addition of H_2O_2 to sperm suspensions. In non-oxidized state, this probe emits red fluorescence while green fluorescence is emitted in peroxidized state. For each sample, 10,000 sperm were counted and the percentage of peroxidized lipids was reported.

2.8. Statistical analysis

Due to overlapping results, the control and sham groups were merged, analyzed, and reported in the results section as a unique control-sham group. Data analysis was performed using the Statistical Package for the Social Sciences for Windows, version 15.0 (SPSS, Inc., Chicago, IL, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA). The data were presented as mean ± standard error of mean (SEM) and $P < 0.05$ was assumed as significant.

3. Results

Two months after varicocele induction, the testis length, width, thickness, and volume, were significantly reduced in varicocelized rats as compared to control-sham rats ($P<0.05$), whereas testis weight were similar (Table 1). Four months after varicocele induction, length, width, and volume of testis significantly worsened in varicocelized rats as compared to those sacrificed at 2 months and to varicocelized rats receiving NAC treatment.

3.1. Sperm parameters and lipid peroxidation

Compared to control-sham group, two months after varicocele induction, varicocelized rats had significantly lower mean sperm concentration (62.80±4.06 vs. 107.05±2.74; P =0.000) and total motility (42.00±2.23 vs. 82.00±1.25; P =0.000), whereas mean percentage of sperm abnormal morphology (9.60±0.33 vs. 7.00±0.41; P =0.01) and lipid peroxidation (39.00±1.51vs. 9.30±1.36; P =0.000) were significantly higher (Figure 2). At four months, mean sperm concentration (83.33±10.67 vs. 44.40±2.40; P =0.000) and total motility (90.50±3.46 vs. 37.80±1.06; P =0.000) were significantly higher, whereas sperm abnormal morphology (8.16±1.54 vs. 12.10±0.76; P =0.02) and lipid peroxidation (12.00±1.86 vs. 68.20±3.96; P =0.000) were significantly lower in varicocelized rats that received NAC, compared to untreated varicocelized rats and similar to those of the control-sham group (Figure 2).

3.2. Sperm chromatin status

As shown in Figure 3 (A), the increase of the mean percentage of sperm DNA damage at 2 months in varicocele induction group compared to the control-sham group, did not reach statistical significance (53.75±5.93 vs. 39.20±3.96; P =0.47). At 4 months, the DNA damage was significantly reduced in NAC treated animals compared to untreated ones (6.25±2.83 vs. 49.20±18.59; P =0.02) and to control-sham group at two months (P =0.027). As shown in Figure 3 (B), at 2 months, the mean percentage of aniline blue positive spermatozoa or abnormal chromatin condensation was significantly increased in varicocele induction group (9.03±1.48), compared to the control-sham group (5.13±0.45; P =0.02). Interestingly, the rate of AB positive sperms also significantly increased (10.20±1.33; P =0.003) in control/sham groups at 4 months, which can be interpreted as a sign of ageing. At 4 months, the mean of aniline blue positive spermatozoa was significantly lower in varicocelized rats that received NAC (3.23±0.89), compared to untreated varicocelized rats (13.23±1.33; P =0.000) and to the control-sham group at four months (10.20±1.33; P =0.002). This indicates that NAC treatment had also reversed the supposed effects of ageing on chromatin condensation.

4. Discussion

It is well established that varicocele-associated infertility and ROS-mediated sperm damage are detectable in 30-80% of cases (16). Therefore, in addition to medication or surgical therapy, supplementation with antioxidants can be considered as a treatment option.

Besides increased oxidative aggression, oxidative stress also associates with reduced antioxidant capacity, such as GSH deficiency (17). GSH, acting as substrate for glutathione peroxidase, scavenges reactive oxygen species, including lipid peroxides (18), and is involved in the regeneration of most of the intracellular antioxidants acting as a redox buffer (19).

Table 1: Comparison of the morphometric characteristics of testis (length, width, thickness, weight, volume) between all the study groups at two and four months

Time	Groups	Testis				
		Length	Width	Thickness	Weight	Volume
2 Months	Control-sham	1.74±0.01^{a}	0.89±0.01^{a}	0.69±0.01^{a}	1.61±0.02	1.62±0.04^{a}
	Vl	1.54±0.04^{a}	0.72±0.02^{a}	0.48±0.02^{a}	1.50±0.05	1.12±0.09^{a}
4 Months	Control-Sham	1.62±0.01^{b}	0.65±0.01^{b}	0.45±0.01	1.53±0.01	1.45±0.03 ^{b}
	Vl.NAC-	1.36±0.02^{bc}	0.48±0.03^{bc}	0.42±0.02	1.44±0.07	1.02±0.14^{bc}
	Vl.NAC+	1.75±0.03^{c}	0.78±0.03^{c}	0.48±0.01	1.61±0.08	1.48±0.04^{c}

*Common letters in each column indicate significant differences between groups at p-value<0.05.

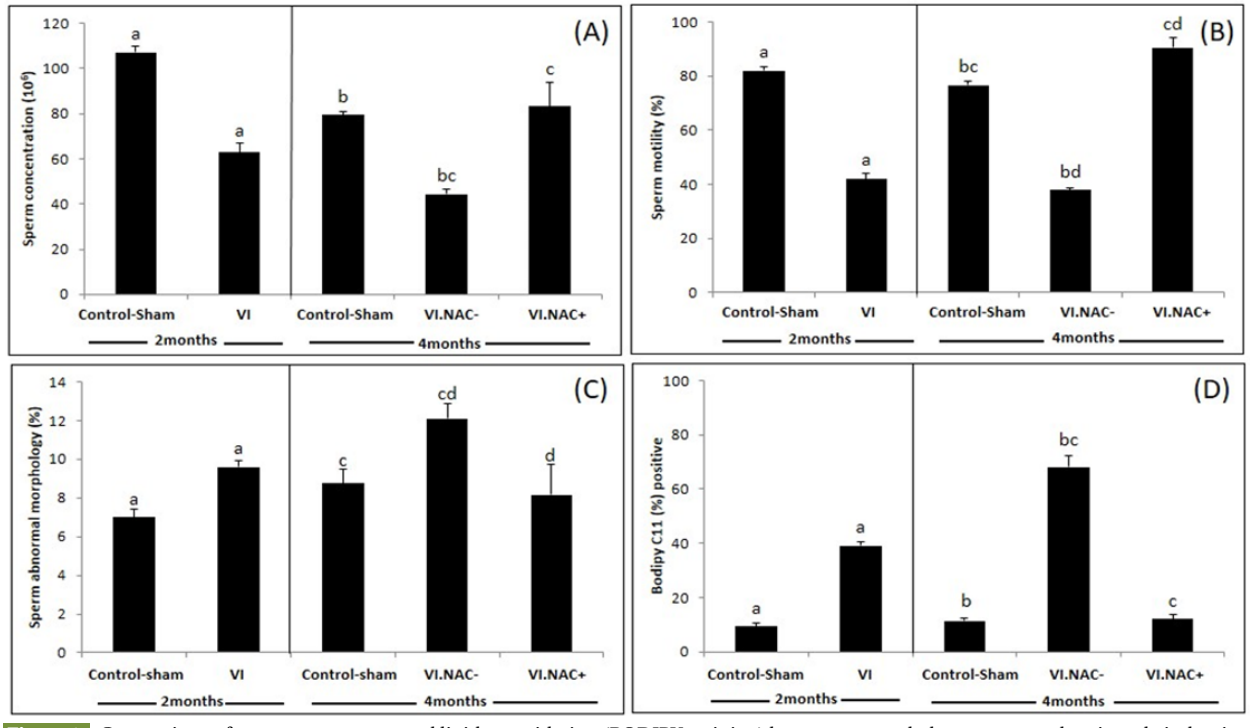


Figure 1: Comparison of sperm parameters and lipid peroxidation (BODIPY staining) between control-sham group and varicocele induction (Vl) group at two months, and also between control-sham, varicocelized rats that received NAC and varicocelized rats that did not receive NAC groups at for months.

At the molecular level, nuclear factor erythroid 2-related factor 2 (Nrf2) acts as a redox-sensitive transcription factor involved in GSH production and regeneration within anti-inflammatory and antioxidant pathways. Nrf2 is connected to cytoplasmic Keap1 (Kelch-like ECH-associated protein 1) and is degraded by ubiquitin-proteasome pathway. When oxidative stress occurs, Keap1 cysteine residues are oxidized and Keap1 gets separated from Nrf2. Then, Nrf2 is transferred into nucleus and stimulates the expression of antioxidant genes, activation of glutathione reductase, and finally, regeneration of GSH. N-acetylcysteine releases cysteines for glutathione biosynthesis and may help to expand the intracellular GSH pool, thus potentiating the mechanism mentioned above. Previous studies showed that NAC could improve Nrf2/GR/GSH pathway in acute lung injury related to oxidative stress (18, 20), and increase the expression of Nrf2 and decrease the level of caspase 3, p53, and apoptosis in renal epithelial tubular cell (21). Accordingly, we assessed the effects of NAC treatment on sperm parameters, chromatin status, and lipid peroxidation, in a rat surgical varicocele model.

Two months after the varicocele induction, morphometric parameters of testis, such as length, width, thickness, and volume, were significantly decreased with the exception of testis weight whose reduction was not significant. Duarte et al. (2010) demonstrated a significant reduction in ratio of round spermatids/pachytenes spermatocytes and an increase in germ cell degeneration during meiosis in varico-

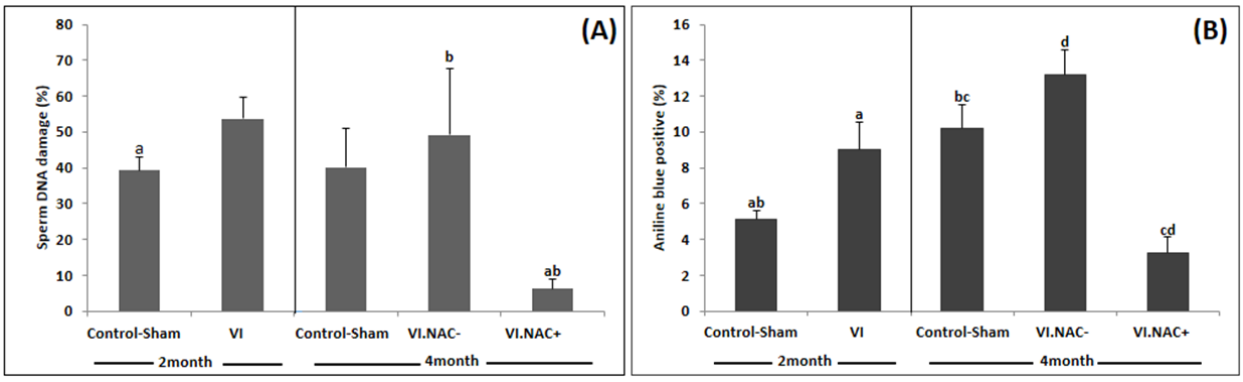
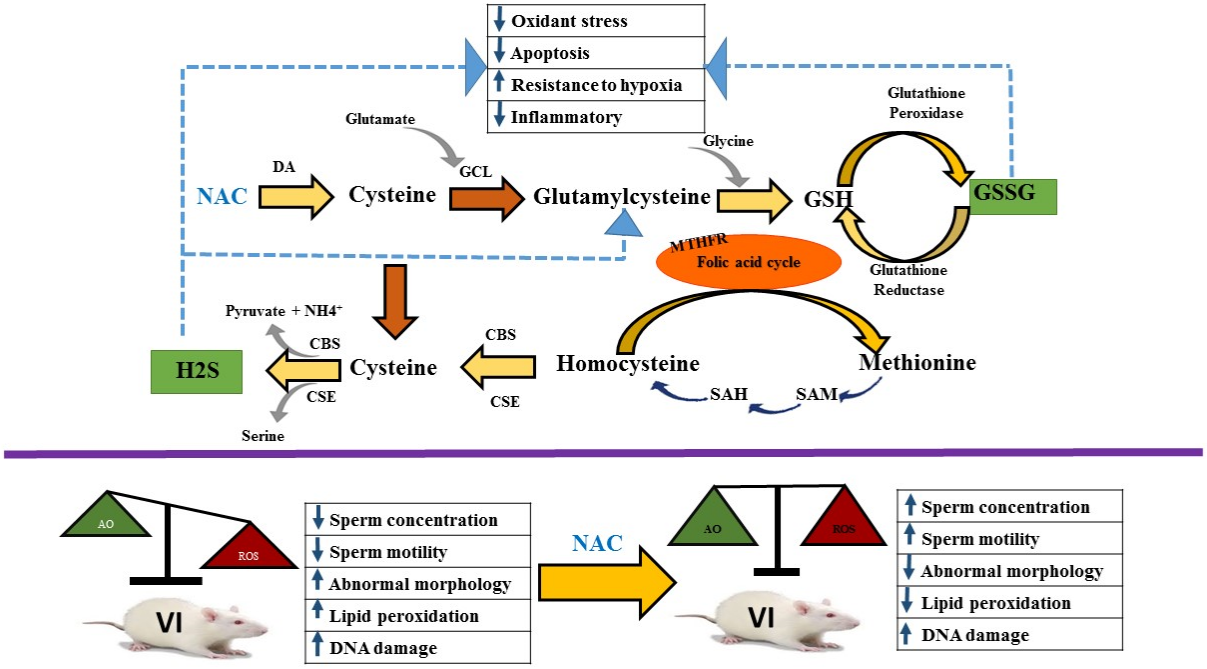


Figure 2: Comparison of percentage sperm DNA damage (A) and aniline blue positive (B) spermatozoa between control-sham group and varicocele induction (VI) group at two months, and also between control-sham, varicocelized rats that received NAC and varicocelized rats that did not receive NAC groups at for months.



ROS: Reactive Oxygen Species, AO: Antioxidant, NAA: N-Acetyl Cysteine, DA: Deacetylation, GCL: Glutamate- Cysteine ligase, GSH: Reduced Glutathione, GSSG: Oxidized Glutathione, CBS: Cistathionine Beta-Synthase, CSE: Cistathionine Gamma- Lyase, SAH: S- adenosylhomocysteine, SAM: S- adenosylmethionine, MTHFR: Methylenetetrahydrofolate reductase , VI: Varicocele induction

Figure 3: N-acetylcysteine (NAC) can improve testicular morphometric parameters and sperm function in the varicocele induction model in the rat via recycling one Carbone cycle.

celized rats (22). These findings, along with our results, add evidence to the hypothesis that varicocele leads to altered testicular function. Also, the parameters of sperm quality, such as concentration, total sperm motility, and morphology, significantly worsened after surgery, confirming the validity of our surgical model of varicocele. These results are consistent with previous studies demonstrating that varicocele can lead to impaired spermatogenesis and spermiogenesis (23, 24). All these damages were associated with an increased oxidative stress, as indicated by the significant increase of lipid peroxidation 2 months after surgery. Our varicocele model also resulted in a significantly decreased mean percentage of aniline blue positive spermatozoa with a concomitant non-significant reduction of DNA damage. Abnormal replacement of histone-to-protamine is known as one of the underlying causes of DNA damage in sperm. In light of this, previous studies showed positive association between the percentage of residual histone and the DNA damage in infertile men (25, 26).

The serious damages resulting from our surgical varicocele

model may be explained by several mechanisms, mostly related to an oxidative aggression, including: 1) high susceptibility of mitochondrial membrane to peroxidation due to its high content of polyunsaturated lipids, which peroxidation of lipids may hamper ATP production, sperm maturation, and motility; 2) leydig cells susceptibility to lipid peroxidation and to impaired transport of cholesterol, precursor for testosterone biosynthesis, into mitochondria leading in decreased testosterone production; 3) low expression of heat shock proteins in response to hyperthermia due to varicocele-induced oxidative stress; 4) consumption of glutathione in scavenging activities, leading to a low ratio of reduced (GSH) to oxidized (GSSG) glutathione, which promotes apoptosis in germ cells. All these mechanisms are likely to apply also to clinical varicocele which is, however, also dependent on other co-factors such as lifestyle (smoking, obesity, and hypertension) as well as the environmental and genetic backgrounds (27-32). After the validation of varicocele induction in rats, we assessed whether the administration of NAC exerts preventive or corrective effects. The treatment with NAC, significantly improved sperm parameters (concentration, total motility, and morphology) and reduced the level of sperm lipid peroxidation and of DNA damage. This is in agreement with Barekat et al. (2016) showing that NAC helps to recover sperm parameters when administered as adjunct therapy post-varicocelectomy, and supports the idea that NAC may also be of benefit as an alternative to surgery (11).

In oxidative conditions NRF2 is released to increase GSH levels via activation GSH biosynthetic pathway as a corrective mechanism, and NAC supplementation may facilitate this process. Rao and Shaha (2002), using a rat model of methoxyacetic acid toxicity, clearly showed that the protective effect of NAC was mediated by replenishment of cellular GSH (33). Moreover, human sperms exposed to Stattic V (Stat3 inhibitory compound V) undergo immobilization with mitochondrial membrane depolarization and massive release of ROS. All these effects where inhibited by NAC (34) which points to an effect on GSH level. Finally, NAC, together with alpha lipoic acid, was shown to revert the endocrine and seminal damages caused by forced intensive swimming in rats which level of ROS was high (30). Furthermore, in this model, the effect of NAC was associated with a correction of the GSH to GSSG ratio. In summary, although we did not measure GSH level, our findings and those already reported in the literature, strongly point to an antioxidant effect of NAC working as thiol donor for GSH synthesis and thereby modulating the intracellular GSH balance.

In the current study, the mean percentage of aniline blue positive spermatozoa and DNA damage significantly increased in varicocelized rats. This finding is in agreement with previous reports on humans and animal models, showing that varicocele is associated with inappropriate exchange of histone-to-protamine, resulting in low quality of semen, high percentage of DNA damage, and global impairment of spermatogenesis (35). In our model, the level of aniline blue positive sperms increased over time in control-sham groups, indicating a possible ageing effect. The treatment with NAC, besides counteracting the damage from varicocele, also prevented such ageing effects, suggesting a specific positive influence on sperm chromosome compaction. Cysteines released by NAC may indeed directly react with homocysteine (the end-product of the one carbon metabolism responsible for DNA and histone methylation as well as initial substrate for cysteine/GSH synthesis) to produce hydrogen sulfide (H_2S), a soluble reducing gas (36). H_2S is an endogenous gasotransmitter that plays pivotal roles in intracellular redox homeostasis (37) and that can be generated only as a by-product of GSH synthesis.

In particular, H_2S can be generated by direct reaction of cysteine with homocysteine catalized by the enzyme Cistathionine Beta Synthase (CBS) that generates H_2S and cistathionine. Cystathionine is then further converted to cysteine by the enzyme cystathionase (CTH) with another H_2S released (38). H_2S produced as such from cysteines, acts as an intracellular activator for Methionine Transferase Reductase (MTRR), thus activating the B12-mediated key step in re-methylation of homocysteine to methionine for the synthesis of S-adenosyl-methionine, the substrate for DNA, and histones methylation (39). DNA and histone methylation is the very initial trigger for protamine transitions (40). It is worth noting that when NAC was used to treat men following varicocelectomy (11), the improvement of sperm nuclear compaction was the main advantage over varicocelectomy alone. Accordingly, the effect on protamination might be the most clinically relevant effect of NAC, both in our model and in clinical varicocele. In this study, we did not assay antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione, after NAC therapy of varicocelized rats. Therefore, further studies are needed to evaluate these enzymes and molecular markers involved in one Carbone cycle.

5. Conclusion

Our model for surgical varicocele in rats resulted in damages to testes and spermatogenesis, resembling those observed in clinical varicocele. The supplementation of varicocelized rats with NAC was effective in antagonizing the damage as well as in preserving testicular structure and spermatogenetic function by recycling one Carbone cycle (Figure 4). Considering that the clinical use of NAC in infertile men did not result in any tolerability issues (7), this supplementation should also be considered for the treatment of varicocele patients. However, the actual dose and duration of the treat-

ment remains to be defined by prospective clinical trials.

6. Appendix

6.1. Acknowledgements

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6.2. Author contribution

Marziyeh Tavalaee: collection and/or assembly of data, data analysis, interpretation, manuscript writing, and final approval of manuscript. Parisa Mohammadi: preparation of samples and tests, collection and/or assembly of data, data analysis. Maurizio Datilio: conception, design, interpretation, and manuscript writing; Mohammad H. Nasr-Esfahani: Conception, design, data analysis, interpretation, manuscript writing and final approval of manuscript.

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6.4. Conflict of interest

The authors of this study declare that they have no conflict of interest.

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