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Ellagic acid attenuates testicular disruption in rheumatoid arthritis via targeting inflammatory signals, oxidative perturbations and apoptosis

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ABSTRACT

Background and purpose: Reduced male fertility has been regarded as a serious complication of rheumatoid arthritis. Phytochemicals have been described as protective agents against rheumatoid arthritis-linked testicular impairment. The current study aimed to investigate the potential protective effects of ellagic acid on rheumatoid arthritis-evoked testicular dysfunction vis-à-vis the reference anti-inflammatory celecoxib.

Experimental approach: Ellagic acid (50 mg/kg/day) and celecoxib (5 mg/kg/day) were administered orally for 20 days in adjuvant-induced arthritic rats.

Key findings: Current data revealed that ellagic acid counteracted rheumatoid arthritis-evoked testicular histopathologic changes, disrupted sperm characteristics and low gonadosomatic index with comparable efficacy to celecoxib. Ellagic acid also enhanced the testicular steroidogenesis via upregulating the gene expression of 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase and steroidogenic acute regulatory protein with consequent boosting of serum testosterone. Notably, ellagic acid attenuated the testicular inflammatory responses through suppression of myeloperoxidase, tumor necrosis factor-α and cyclo-oxygenase-2 protein expression together with enhancing the anti-inflammatory signal interleukin 10. Ellagic acid also curbed the redox alterations via lowering the production of lipid peroxides and nitric oxide and elevation of the anti-oxidant reduced glutathione. In support of cell survival, ellagic acid combated testicular apoptosis through down-regulating caspase-3 protein expression.

Significance: The present work accentuates the beneficial actions of ellagic acid in rheumatoid arthritis-incurred testicular impairment and disrupted spermatogenesis via combating the inflammatory, oxidative and apoptotic aberrations.

1. Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammatory events that incur damage chiefly to the synovial joints, leading to joint abnormality and disability. In addition, extra-articular manifestations of RA have been defined, particularly, the testicular impairment, disrupted testosterone production and impotence in men with long-standing RA [1]. In this regard, the chronic activation of immune cells drives the production of diverse pro-inflammatory signals that undermine the testicular steroidogenesis and testosterone production, which may even predispose to severe RA flares [2]. Additionally, these inflammatory events directly impact the testicular architecture, provoking decreased testicular weights, disrupted sperm production and functions [3]. Oxidative stress associated with excessive production of reactive oxygen species (ROS) has been characterized as a central player in RA-incurred testicular dysfunction and impaired semen quality [4]. According to Li et al. [5], Freund's complete adjuvant (FCA)-triggered ROS production is well recognized as a detrimental event to the testicular function. Oxidative stress encourages germ cell apoptosis, damages sperm DNA and impairs sperm movement to fertilize the oocyte [3].

Freund's complete adjuvant-induced arthritis in rats has been

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; AA, Adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid; FCA, Freund's complete adjuvant; GAPDH, glyceraldehyde-3-P dehydrogenase; GSH, reduced glutathione; IL-10, interleukin 10; MDA, malondialdehyde; MPO, myeloperoxidase; NO, nitric oxide; RA, rheumatoid arthritis; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein; TNF-α, tumor necrosis factor-α

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envisioned as a widely-used model for human RA to study its pathogenesis, including the underlying mechanisms of RA-associated testicular dysfunction [6–8]. In this context, declined serum testosterone together with altered luteinizing hormone (LH) levels have been described in rats with adjuvant arthritis [7].

Ellagic acid, a natural polyphenolic phytochemical, is found in diverse plant species including strawberries, pomegranate, grape, blackberries as well as raspberries. Ample evidence revealed that ellagic acid has exerted diverse hepato-, nephro-, neuro- and cardio-protective actions thanks to its marked anti-oxidant and anti-inflammatory features [9,10]. Ellagic acid has been reported to directly scavenge ROS e.g., hydrogen peroxide and hydroxyl radical as well as reactive nitrogen species (RNS) e.g., peroxynitrite. Alternatively, ellagic acid can indirectly impact the redox milieu via augmenting the cellular anti-oxidant levels [10]. Evolving evidence has demonstrated that ellagic acid can protect the male reproductive system against several toxicant-induced testicular pathologies via counteracting testicular redox alterations [11,12], inflammatory responses [11] and apoptotic events [12]. Despite these protective actions, the potential of ellagic acid to alleviate RA-induced testicular impairment has not been previously explored. Thus, the aim of the present study was to explore the protective potential of ellagic acid along with the underlying mechanisms against testicular impairment in rats with adjuvant arthritis.

2. Materials and methods

2.1. Ethics statement

The animal experimentation concurred with the instructions of the US NIH Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996). The current protocol was approved by the Research Ethics Committee for Experimental and Clinical Studies at National Organization for Drug Control and Research (NODCAR; approval number: NODCAR/I/14/19).

2.2. Animals

Healthy adult male Sprague–Dawley rats (200 ± 20 g; NODCAR, Giza, Egypt) were kept at controlled surroundings of temperature (22 ± 2 °C), relative humidity (60 ± 10%) and a 12/12 h light–dark schedules and ad libitum supply of standard chow diet and water. An acclimatization period of 2 weeks was allowed before the start of animal experimentation.

2.3. Chemicals and reagents

Freund complete adjuvant (FCA; cat. no. F5881) in concentration of 1 mg/mL and ellagic acid (cat. no. E2250) were procured from Sigma-Aldrich, St. Louis, MD, USA whereas celecoxib was a kind gift from Pfizer, Cairo, Egypt. All remaining reagents and fine chemicals were of analytical grade and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless specified herein.

2.4. Experimental design

Forty rats were divided randomly into four groups (10 rats per group):

I) Control group: normal rats which received 0.5% carboxymethyl cellulose oral vehicle and were inoculated with saline into the paw/tail root.
II) Arthritic group (AA): Arthritic-non treated animals which received 0.5% carboxymethyl cellulose for 20 days. To induce arthritis, 0.1 ml of Freund's complete adjuvant (FCA) was injected subcutaneously (sc) into the plantar skin of the left hind paw. Another two booster subcutaneous doses of 0.1 ml FCA were given into the root of the tail at 1 h and 24 h post FCA inoculation to enhance its systemic effect [3,13–15].
III) Celecoxib-treated group (Arthritic + CLX): Arthritic rats that received the reference celecoxib (5 mg/kg/day, orally) for 20 days starting 1 h after FCA inoculation [16,17].
IV) Ellagic acid-treated group (Arthritic + ELA): Arthritic rats that received ellagic acid (50 mg/kg/day, orally) for 20 days starting 1 h after FCA inoculation [16,18].

The vehicle, ellagic acid and celecoxib were orally administered by gavage in a final volume of 1 ml per 200 g rat, in the morning time (8:00–9:00 a.m.).

On the final day of the experiment, animals were weighed and the blood samples were collected from the retro-orbital plexus under anesthesia with sodium phenobarbital (150 mg/kg, i.p.). Serum was separated and used for the assessment of testosterone level. Then, rats were sacrificed and the testes were immediately collected and weighed. The cauda epididymides were harvested in phosphate-buffered saline (pH 7.2) for the investigation of the sperm parameters. After decapsulation, the right testis was separated into two sections and kept at −80 °C. One part was homogenized in ice cold saline for the estimation of the biochemical parameters and the other part was used for examination of the gene expression of the key steroidogenesis enzymes. The left testis was kept at 10% neutral-buffered formalin for histopathologic/immunohistochemical studies.

2.5. Assessment of arthritis

Arthritis was monitored by sequential measuring of the paw edema for 3 weeks as described [14]. In this regard, the ankle dorso-plantar diameter was obtained by means of vernier caliper. For each rat, the ankle diameter value of FCA pre-injection was used as the baseline. The changes of the paw diameter were recorded and presented in millimeters.

2.6. Evaluation of sperm count, motility and abnormality

For the determination of sperm count, motility and abnormality, the epididymis was minced with scissors in phosphate-buffered saline at 37 °C as described by Bearden and Fuquay [19].

2.7. Gonadosomatic index

The body weight and the weight of the testes were recorded at the end of the experiment. The following formula was used to calculate the gonadosomatic index (or the relative testicular weight): (testes weight/body weight) × 100 [3].

2.8. Estimation of serum testosterone level

The quantitative determination of serum testosterone levels was carried out by means of a rat-specific competitive ELISA kit (Cusabio, PRC) as instructed by the manufacturer. The absorbance was recorded on a microplate reader at 450 nm.

2.9. Enzymatic markers of testicular function

The AST, ALT, ACP and ALP markers of testicular function were determined in the testicular homogenates using specific colorimetric kits (Biodiagnostic, Cairo, Egypt) based on the instructions of the manufacturer. The protein content of the testis homogenate was determined by the sensitive assay of Lowry et al. [20].

2.10. Determination of testicular myeloperoxidase (MPO) activity

The activity of MPO is considered as a sensitive marker of

neutrophil invasion to the tissue [21]. The assay was performed as described by Krawisz et al. [22]. Three freezing/thawing cycles and the hexadecyl-trimethyl-ammonium bromide detergent were used to ensure the extraction of the MPO enzyme. Color was developed with the aid of 0.167% *o*-dianisidine hydrochloride and 0.0005% H_2O_2 in potassium phosphate buffer (50 mmol/L, pH 6) and the absorbance was recorded at 460 nm.

2.11. Determination of testicular oxidative stress (lipid peroxides, nitric oxide and reduced glutathione) and inflammatory biomarkers (TNF-α and IL-10)

The levels of lipid peroxides, expressed as malondialdehyde (MDA), were assayed according to the method described by Beuge and Aust [23]. The color was developed by reaction with thiobarbituric acid (TBA)/HCl reagent and the pink color was measured at 535 nm (UV-120 Shimadzu spectrophotometer, Japan). For the determination of reduced glutathione (GSH), the homogenate was deproteinized with 5% sulfosalicylic acid and the resultant supernatant was used for the GSH assay as described by Beutler et al. [24]. The color developed by 5,5′-dithiobis (2-nitrobenzoic acid) addition was measured colorimetrically at 412 nm. The levels of nitric oxide were assayed using specific colorimetric kit from Biodiagnostic (Cairo, Egypt) [25], whereas the levels of TNF-α were determined with Cusabio ELISA kit (cat. no. CSB-E11987r, Cusabio, PRC) which has a 6.25–400 pg/ml detection range. Likewise, an ELISA kit specific for IL-10 (cat. no. CSB-E04595r, Cusabio, PRC) with detection range of 3.12–200 pg/ml was used for determination of IL-10 levels. The manufacturer's instructions were strictly followed for both kits.

2.12. Quantitative RT-PCR of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and steroidogenic acute regulatory protein (StAR) gene expression

Total RNA was extracted from rat testes with Qiagen tissue extraction Kit (cat. no. 74104; Qiagen, USA) as per the protocol of the manufacturer. The purity of extracted RNA was checked by the absorbance at 260/280 ratio (for protein) and 260/230 (for salts and solvents). RNA was used for preparation of complementary DNA (cDNA) using Superscript choice systems (cat. no. 18090019; Life Technologies, Breda, Netherlands) according to the manufacturer's procedure and all products were stored at −20 °C. A negative control (no enzyme) was performed in the reverse transcription, in order to exclude the DNA contamination during RNA extraction procedures. The Gene expressions were analyzed by quantitative RT-PCR using an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) and a SYBR green PCR master mix (cat. no. 4309155; Applied Biosystems, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The sense and anti-sense primers are described in Table 1. The thermal cycling conditions consisted of initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The specificity of each amplified reaction was verified by a dissociation curve where a single peak was observed using all primer pairs. All these steps comply with the MIQE guidelines [26].

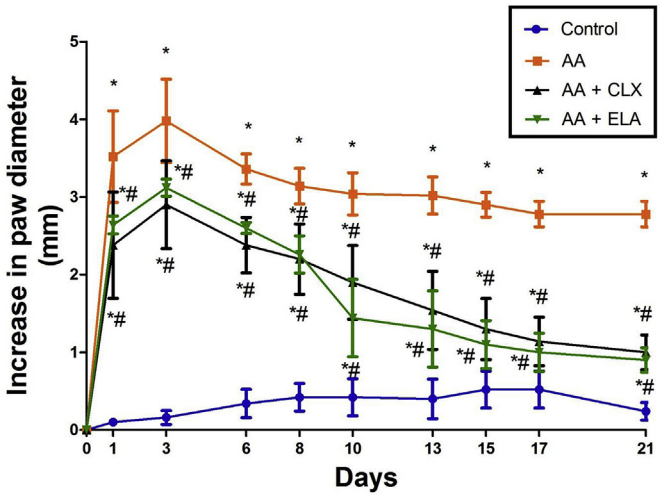

Fig. 1. Ellagic acid and celecoxib suppress paw edema in adjuvant arthritic rats. Arthritis was induced by sc injection of 0.1 ml of Freund's complete adjuvant (FCA; day 0) at a concentration of 1 mg/ml into the right hind paw plus two additional intra-dermal injections of FCA (0.1 ml) at the tail root, 1 h and 24 h, following paw FCA injection. Ellagic acid (50 mg/kg/day) and celecoxib (5 mg/kg/day) were orally administered, starting one day after paw FCA inoculation on day 0 and continued till day 20. Values represent mean ± SD (n = 10). * Significant difference versus control gp at $p < 0.05$ and # Significant difference versus arthritic gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

2.13. Histopathologic examination and microscopic damage scoring

Testes from all the experimental groups were harvested and were fixed in 10% neutral-buffered formalin and were processed for paraffin sectioning [27]. Three-micron sections were prepared by slidge microtome and were stained with hematoxylin and eosin (H &E) to evaluate the histologic alterations under light microscope (Olympus CX21, Japan) fitted with camera. The identity of samples was not disclosed to the observer to avoid bias.

The microscopic damage pertaining to the degeneration of spermatogonial cells lining the seminiferous tubules, interstitial edema and congestion of blood vessels was scored on a 0 to 3-point scale: 0 (no change), 1 (mild change), 2 (moderate change) and 3 (severe change). The damage scores were represented as medians with interquartile range.

2.14. Immunohistochemical detection of cyclo-oxygenase-2 (COX-2) and caspase-3

The detection of testicular COX-2 and caspase-3 protein expression was determined using immunohistochemistry [28]. The tissue sections were deparafinized in xylene and rehydrated in descending

Table 1
Primers used for the real-time RT-PCR.

mRNA species	Primer sequence	Gene accession No.	Amplicon Size (bp)	Primer efficiency
3β-HSD	Forward 5′-TGTGCCAGCCTTCATCTAC-3′ Reverse 5′-CTTCTCGGCCATCCTTTT-3′	NM_001007719.3	145	97%
17β-HSD	Forward 5′-GACCGCCGATGAGTTTGT-3′ Reverse 5′-TTTGGGTGGTGCTGCTGT-3′	NM_054007	140	99%
StAR	Forward 5′-GGGCATACTCAACAACCAG-3′ Reverse 5′-ACCTCCAGTCGGAACACC-3′	NM_031558	111	101%
GAPDH	Forward 5′- CTCCCATTCTTCCACCTTTG -3′ Reverse 5′- CTTGCTCTCAGTATCCTTGC -3′	NC_005103.4	295	95%

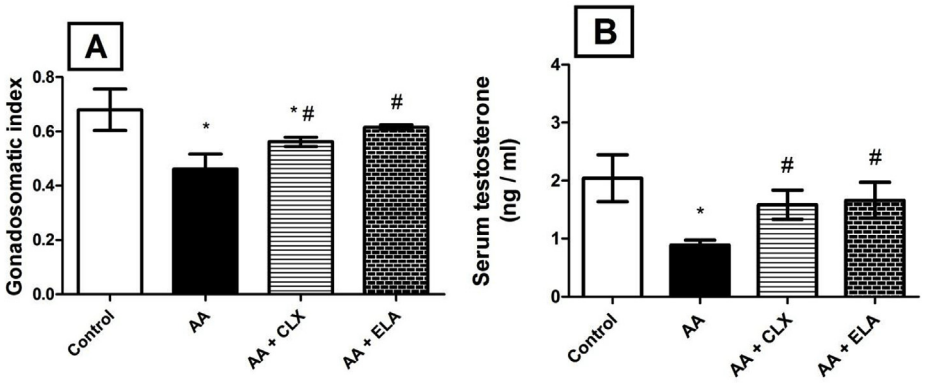

Fig. 2. Ellagic acid and celecoxib boost the gonadosomatic index (A) and serum testosterone (B) in adjuvant arthritic rats. Serum and testes were collected on day 21 post FCA inoculation. The induction of arthritis and the treatment protocol were described in Fig. 1 caption. Values represent mean ± SD (n = 10). * Significant difference versus control gp at $p < 0.05$ and # Significant difference versus arthritic gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

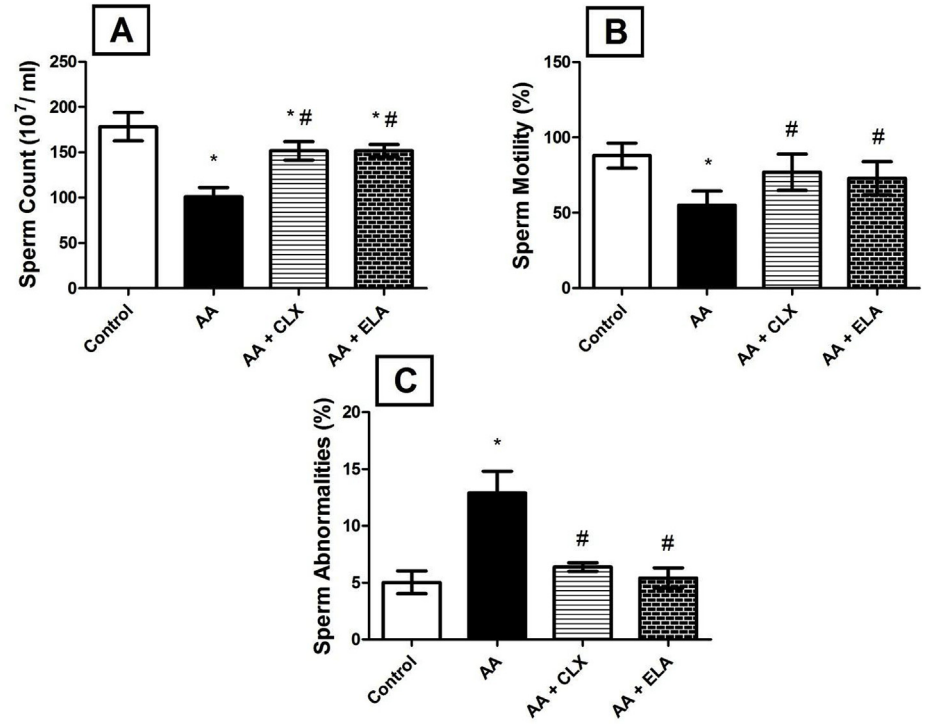

Fig. 3. Ellagic acid and celecoxib modulate sperm characteristics in adjuvant arthritic rats. (A) Sperm count. (B) Sperm motility. (C) Sperm abnormalities. The induction of arthritis and the treatment protocol were described in Fig. 1 caption. The seminal fluid was collected on day 21 post FCA inoculation. Values represent mean ± SD (n = 10). * Significant difference versus control gp at $p < 0.05$ and # Significant difference versus arthritic gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

Table 2
Effect of ellagic acid and celecoxib on the markers of testicular function.

Testicular markers (U/mg protein)	Control	AA	AA + CLX	AA + ELA
AST	73.4 ± 3.8	109.2 ± 11.8*	82.91 ± 3.58#	72.97 ± 4.3#
ALT	50.18 ± 2.6	68.45 ± 8.5*	53.84 ± 3.2#	58.29 ± 5.8#
ACP	228.1 ± 16.7	178 ± 19.8*	226.1 ± 24.5#	222.3 ± 11.4#
ALP	178.1 ± 28.2	118.7 ± 23.4*	178.6 ± 17.5#	170.9 ± 12.4#

The induction of arthritis and the treatment protocol were mentioned in Fig. 1 caption. Testes were collected on day 21 post FCA inoculation. * Significant difference versus control gp at $p < 0.05$ and # Significant difference versus arthritic gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

concentrations of ethanol. Subsequently, the sections were boiled in 0.01 M citrate buffer (pH 6) to retrieve antigen and incubated with 0.3% H_2O_2 to block endogenous peroxidase. Samples were incubated with anti-COX-2 (cat. no. MA5-14568 at 1:200 dilution, Thermo Fisher Scientific, Fremont, USA) or caspase-3 (cat. no. MA1-16843 at 1:200 dilution, Thermo Fisher Scientific, Fremont, USA), followed by incubation in secondary antibody-labeled with HRP. The immunostaining was visualized with 0.02% diaminobenzidine (DAB) and images were acquired with light microscope (Leica Microsystems, Germany). The digital images were quantified with the aid of Image J software (Bethesda, MD, USA).

2.15. Statistical analysis

The parametric data were expressed as mean ± SD. Statistical evaluation of these results was executed by means of one-way analysis of variance (ANOVA). When statistical significance among groups was detected, the post-hoc Tukey-Kramer test was used. The microscopic damage scores (non-parametric) were expressed as median and were analyzed using Kruskal-Wallis analysis of variance, followed by rank-based Mann–Whitney *U* test. The statistical analysis was performed with the aid of GraphPad Prism software (San Diego, CA, USA). p values < 0.05 were regarded as significant.

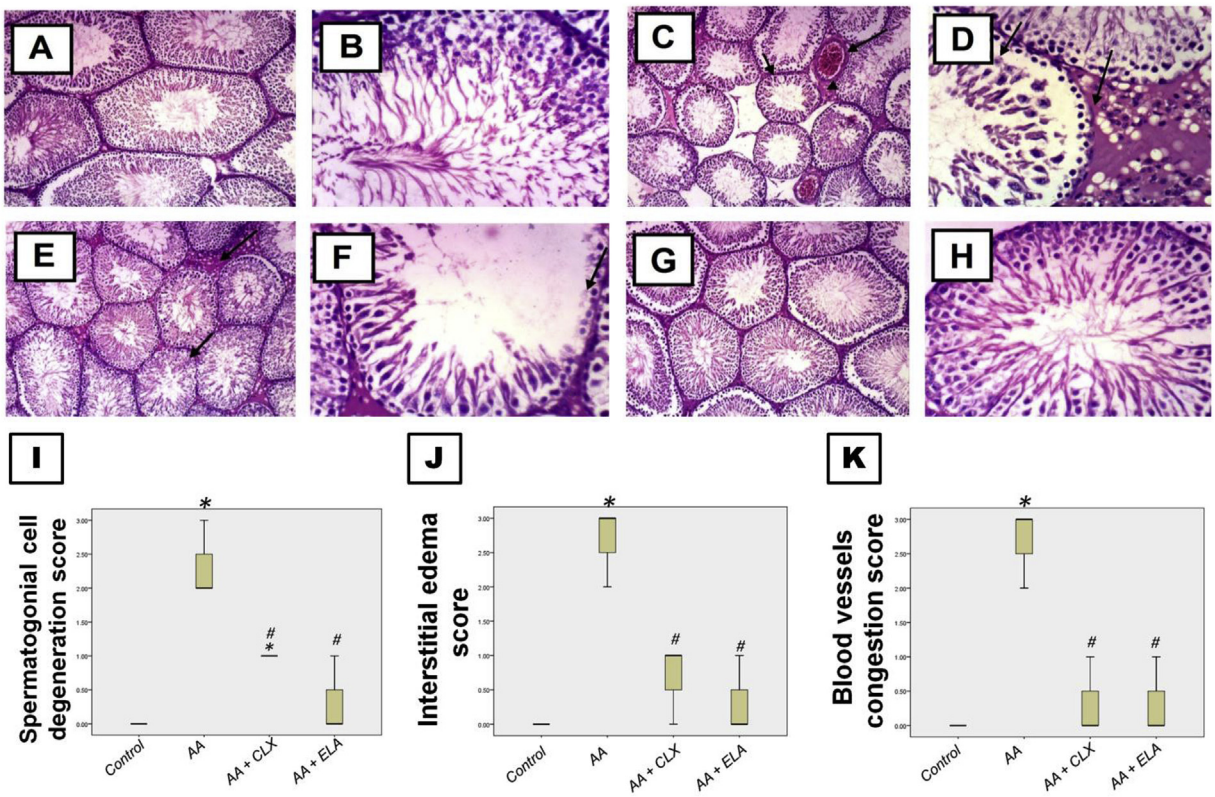

Fig. 4. Ellagic acid and celecoxib attenuate the histopathologic aberrations in the testicular tissue of adjuvant arthritic rats. Representative images of H&E staining demonstrating sections from testes collected on day 21 post FCA paw inoculation (at 100 × and 400 × magnification, respectively, for each group). The induction of arthritis and the treatment protocol were described in Fig. 1 caption. (A,B) Testicular sections of control rats showing mature seminiferous tubules containing complete spermatogenic arrangement. (C,D) Testicular sections of arthritic non-treated rats showing degenerative changes in the spermatogonial cells which line the seminiferous tubules (short arrow) accompanied with blood vessels congestion (long arrow) and interstitial edema (arrow head). (E,F) Testicular sections of arthritic rats treated with celecoxib demonstrated attenuated testicular histopathologic alterations. (G,H) Testicular sections of arthritic rats treated with ellagic acid show preservation of normal testicular architecture. (I) Spermatogonial cell degeneration score. (J) Interstitial edema score. (K) Blood vessel congestion score. The microscopic damage scores are expressed as median with interquartile range. * Significant difference versus control gp at $p < 0.05$ and # Significant difference versus arthritic gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

3. Results

3.1. Ellagic acid and celecoxib suppress paw inflammation and enhance the gonadosomatic index and serum testosterone in rats with adjuvant arthritis

The administration of ellagic acid effectively suppressed the development of paw edema with maximum efficacy on the 21st day as evidenced by 64% inhibition of paw edema in adjuvant arthritic animals (Fig. 1). Of note, the amelioration of paw edema by ellagic acid was similar to that afforded by the reference celecoxib.

More important, arthritic rats suffered decreased gonadosomatic index and serum testosterone levels to 67.8% and 43.5%, respectively, when compared to control rats (Fig. 2). Ellagic acid alleviated these alterations, pointing to its efficacy in attenuating RA-induced testicular damage. It is noteworthy that the effect of ellagic acid was comparable to the reference celecoxib in terms of enhancing the gonadosomatic index and serum testosterone level.

3.2. Ellagic acid and celecoxib improve the sperm characteristics in rats with adjuvant arthritis

We next evaluated the potential protective effects of ellagic acid and celecoxib against spermatogenesis disruption in adjuvant arthritic rats. FCA elicited an obvious disruption of the spermatogenesis process evidenced by decreased sperm count and motility by 43.4% and 37%, respectively and increased incidence of sperm abnormalities by 2.55 fold as compared to the control group (Fig. 3). Ellagic acid increased the sperm count as well as motility by 50.6%, and 32.7%, respectively, and suppressed sperm abnormalities, pointing to its efficacy in combating spermatogenesis disruption.

3.3. Ellagic acid and celecoxib modulate the testicular function biomarkers in rats with adjuvant arthritis

The RA-associated testicular injury was further verified via assaying the enzymatic markers of testicular function, where arthritic rats displayed an increase of testicular AST and ALT activities by 1.48 and 1.36 folds, respectively, with concomitant reduction in ACP and ALP activities by 0.78 and 0.66 folds, respectively, as compared to the control group (Table 2). Ellagic acid and celecoxib reversed these alterations, indicating the alleviation of testicular disturbance provoked by RA.

3.4. Ellagic acid and celecoxib mitigate adjuvant-induced testicular histopathologic alterations

The histopathologic assessment of control group indicated a typical testicular architecture of mature active seminiferous tubules lined with uniformly arranged spermatogenic cells in variable stages of maturation in addition to Leydig and Sertoli cells (Fig. 4A, B). In the same context, microscopic examination of testicular sections from arthritic rats revealed increased damage scores demonstrating marked degenerative changes of the spermatogonial cells lining the seminiferous tubules along with interstitial edema and congestion in blood vessels of tunica albuginia (Fig. 4C, D and 4I-K). Celecoxib attenuated the damage scores

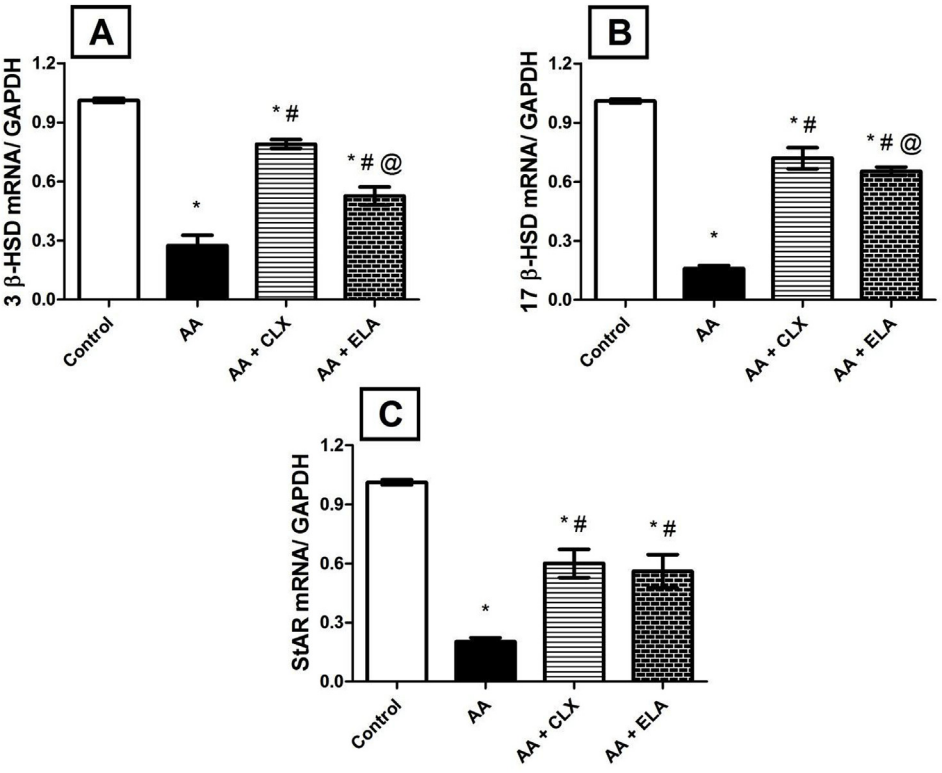

Fig. 5. Ellagic acid and celecoxib enhance the gene expression of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD; A), 17β-hydroxysteroid dehydrogenase (17β-HSD; B) and steroidogenic acute regulatory protein (StAR; C) in the testes of adjuvant arthritic rats. The induction of arthritis and the treatment protocol were mentioned in Fig. 1 caption. Testes were collected on day 21 post FCA inoculation. Values represent mean ± SD (n = 10). * Significant difference versus control gp at $p < 0.05$, # Significant difference versus arthritic gp at $p < 0.05$ and @ Significant difference versus arthritic rats treated with celecoxib gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

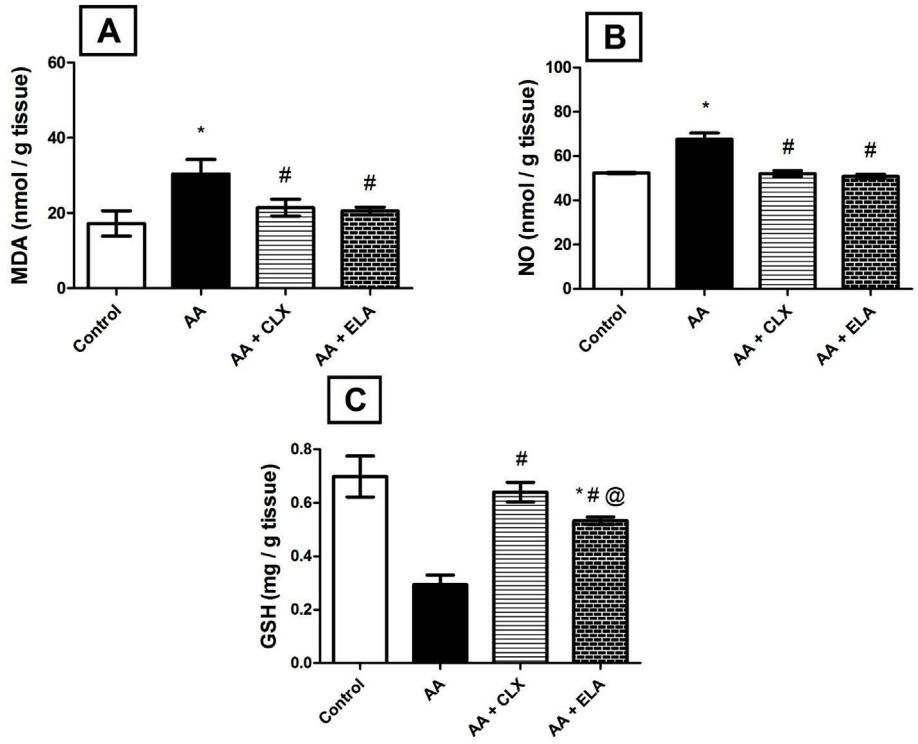

Fig. 6. Ellagic acid and celecoxib alleviate testicular oxidative stress markers in adjuvant arthritic rats. Lipid peroxides expressed as malondialdehyde (MDA; A), nitric oxide (NO; B) and reduced glutathione (GSH; C) were determined in the testicular homogenates of rats with adjuvant arthritis. The induction of arthritis and the treatment protocol were described in Fig. 1 caption. Testes were collected on day 21 post FCA inoculation. Values represent mean ± SD (n = 10). * Significant difference versus control gp at $p < 0.05$, # Significant difference versus arthritic gp at $p < 0.05$ and @ Significant difference versus arthritic rats treated with celecoxib gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

and mitigated the histopathologic alterations with preservation of the testicular architectural integrity and reinstatement of spermatogenesis in the majority of seminiferous tubules (Fig. 4E, F and 4I-K). Likewise, administration of ellagic acid lowered the damage scores and restored the normal structure of mature/active seminiferous tubules that showed complete spermatogenic series in the tubular lumen (Fig. 4G, H and 4I-K).

3.5. Ellagic acid and celecoxib upregulate the gene expression of 3β-HSD, 17β-HSD and StAR steroidogenesis genes

To explore the possible mechanisms underlying the impaired testosterone biosynthesis in arthritic rats, the mRNA expression of 3β-HSD, 17β-HSD and StAR genes was studied using real-time RT-PCR. As shown in Fig. 5, the mRNA expression of 3β-HSD, 17β-HSD and StAR steroidogenesis genes was lowered by 72.9%, 84.1% and 80%,

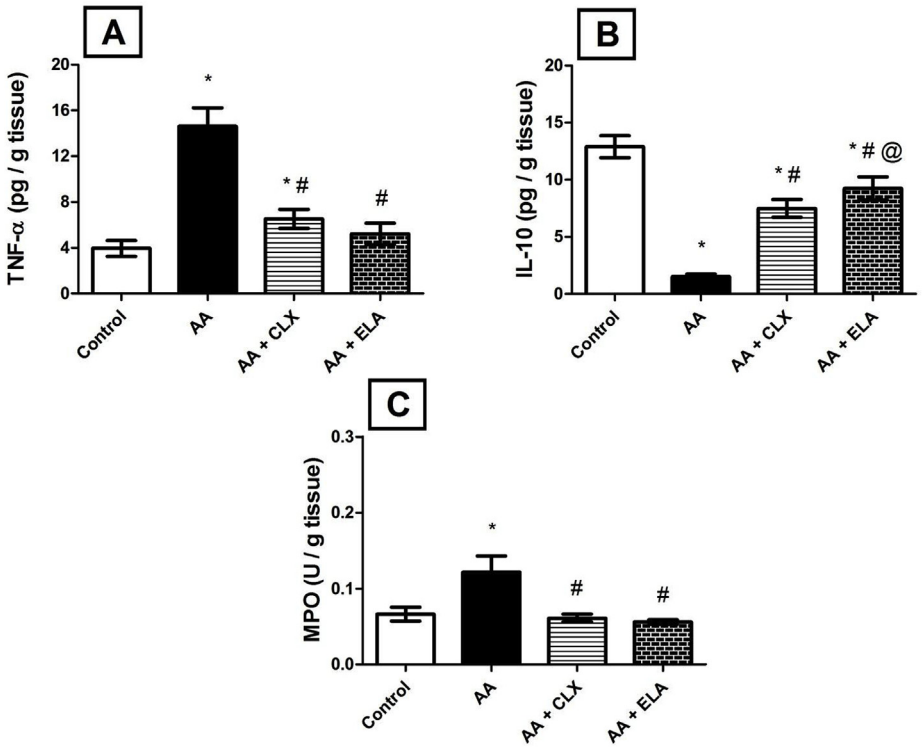

Fig. 7. Ellagic acid and celecoxib suppress arthritis-triggered inflammatory response in adjuvant arthritic rats. The levels of tumor necrosis factor-α (TNF-α; A) and interleukine-10 (IL-10; B) as well as the activity of myeloperoxidase (MPO; C) were determined in the testicular homogenates of arthritic rats. The induction of arthritis and the treatment protocol were described in Fig. 1 caption. Testes were collected on day 21 post FCA inoculation. Values represent mean ± SD (n = 10). * Significant difference versus control gp at $p < 0.05$, # Significant difference versus arthritic gp at $p < 0.05$ and @ Significant difference versus arthritic rats treated with celecoxib gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid.

respectively. Notably, ellagic acid and celecoxib reinstated the expression of these genes, implicating the modulation of these steroidogenesis genes (Fig. 5) and testosterone enhancement (Fig. 2) for combating RA-incurred testicular impairment.

3.6. Ellagic acid and celecoxib curb the oxidative stress markers and potentiate the anti-oxidant milieu

To check the oxidative milieu, we detected lipid peroxides expressed as malondialdehyde (MDA) and nitric oxide (NO) besides reduced glutathione (GSH) anti-oxidant in the testicular tissue of arthritic rats. Freund's complete adjuvant provoked an enhanced oxidative stress evidenced by increased levels of MDA (176%) and NO (129%) along with concomitant decline of testicular GSH (42%) as compared to the control group (Fig. 6). Ellagic acid and celecoxib significantly diminished MDA and NO levels and reinstated testicular GSH in contrast to arthritic animals. These findings indicate that ellagic acid- and celecoxib-induced suppression of oxidative perturbations is engaged in the attenuation of testicular injury in arthritic animals.

3.7. Ellagic acid and celecoxib modulate inflammatory cytokines, myeloperoxidase, and testicular COX-2 protein expression

To examine the inflammatory status of arthritic animals, we checked the testicular MPO, TNF-α and IL-10 along with the expression of COX-2 in the testicular tissue. Rats with adjuvant arthritis displayed elevated MPO (1.8 folds) and TNF-α (3.7 folds) in addition to a marked decline of testicular IL-10 by 88.2%, in contrast to the control group (Fig. 7). These inflammatory alterations were counteracted by ellagic acid and celecoxib administration.

Regarding the testicular COX-2 protein expression, sections from testes of arthritic rats demonstrated an intensive expression that was mainly detected within the interstitial stroma neighboring the basement membrane of the seminiferous tubules (Fig. 8). Ellagic acid and celecoxib administration markedly attenuated COX-2 protein expression in the testicular tissues. Together, these observations suggest that ellagic acid and celecoxib modulation of the inflammatory signals and COX-2 expression are involved in combating RA-associated testicular injury.

3.8. Ellagic acid and celecoxib suppress testicular caspase-3 protein expression in arthritic rats

We also assessed the protein expression of caspase-3 as a trusted marker for testicular apoptosis in adjuvant arthritic rats. Arthritis triggered a significant testicular apoptosis evidenced by increased caspase-3 expression as compared to the control animals (Fig. 9). Noticeably, ellagic acid and celecoxib lowered the enhanced expression of caspase-3 pro-apoptotic signal which favored germ cell survival and protection against RA-linked testicular insult in adjuvant arthritic rats.

4. Discussion

Rheumatoid arthritis is a well-known autoimmune disorder that adversely impacts the male reproductive system triggering impaired fertility, testicular dysfunctions and sperm anomalies along with diminished bioavailable testosterone [1,29]. These events are majorly driven via immune-mediated overshooting of the inflammatory mediators, oxidative and nitrosative stress and germ cell apoptosis [1,3]. The present study reveals, for the first time, that ellagic acid attenuates the testicular impairment in adjuvant arthritic rats as evidenced by improvement of serum testosterone and sperm quality/quantity and lowered testicular microscopic damage. These promising effects were mediated by ellagic acid-induced suppression of the pro-inflammatory signals, oxidative stress and germ cell apoptosis that ultimately boosted steroidogenesis (Fig. 10). Interestingly, ellagic acid demonstrated a similar efficacy to the reference celecoxib for alleviating the testicular disruption in adjuvant-induced arthritis, thus, ellagic acid may offer a safer option for the management of RA-associated testicular dysfunction with fewer side effects. Of note, a previous study by our research group has reported the anti-arthritic efficacy of ellagic acid in the immune-mediated adjuvant-induced arthritis model of RA via down-regulating the pro-inflammatory signals that ultimately attenuated the joints destruction [16].

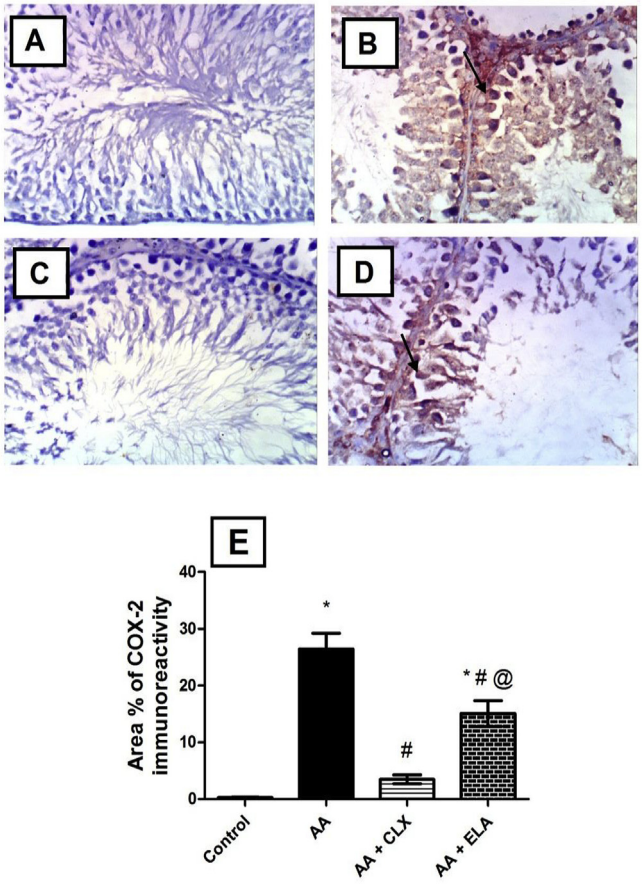

Fig. 8. Ellagic acid and celecoxib downregulate COX-2 protein expression in the testicular tissue of adjuvant arthritic rats. Representative photomicrographs of sections from testes collected on day 21 post FCA paw inoculation (× 400) showing immunohistochemical detection of COX-2 expression. The induction of arthritis and the treatment protocol were described in Fig. 1 caption. (A) Testes of control rats show minimal immunostaining. (B) Testes of arthritic rats show massive immunostaining (brown color). (C) Testes of celecoxib-treated rats show attenuated immunostaining. (D) Testes of ellagic acid-treated rats show attenuated immunostaining. (E) Quantification of COX-2 immuno-reactivity. * Significant difference versus control gp at $p < 0.05$, # Significant difference versus arthritic gp at $p < 0.05$ and @ Significant difference versus arthritic rats treated with celecoxib gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

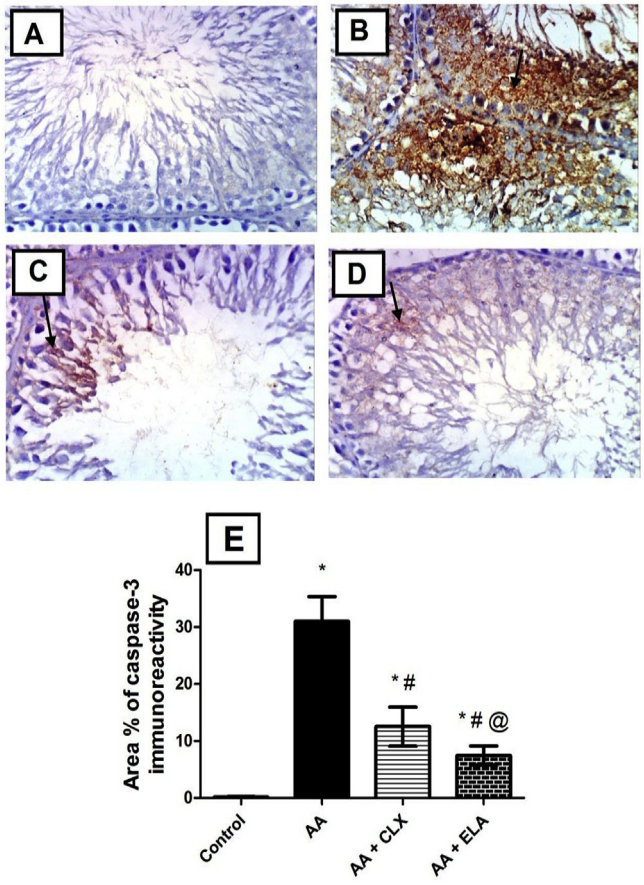

Fig. 9. Ellagic acid and celecoxib downregulate caspase-3 protein expression in the testicular tissue of adjuvant arthritic rats. Representative photomicrographs of sections from testes collected on day 21 post FCA paw inoculation (× 400) showing immunohistochemical detection of caspase-3 expression. The induction of arthritis and the treatment protocol were described in Fig. 1 caption. (A) Testes of control rats show minimal immunostaining. (B) Testes of arthritic rats show intense immunostaining (brown color). (C) Testes of celecoxib-treated rats show lowered immunostaining. (D) Testes of ellagic acid-treated rats show lowered immunostaining. (E) Quantification of caspase-3 immuno-reactivity. * Significant difference versus control gp at $p < 0.05$, # Significant difference versus arthritic gp at $p < 0.05$ and @ Significant difference versus arthritic rats treated with celecoxib gp at $p < 0.05$. AA, adjuvant-induced arthritis; CLX, celecoxib; ELA, ellagic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In the present study, several histopathologic aberrations were detected confirming a marked testicular injury. A noticeable protective effect of ellagic acid, at dose of 50 mg/kg, against the histo-architectural structure of seminiferous tubules and spermatogonial cell degeneration. These results concurred with earlier reports that demonstrated the ameliorative effect of ellagic acid on diverse germ cell degenerative changes and impaired spermatogenesis in sodium valproate- [30] arsenic- [18], and adriamycin- [11,12] evoked testicular injury. In the same context, adjuvant arthitic rats demonstrated a disruption of the enzymatic markers of testicular function e.g., ACP and ALP whose activities were lowered by FCA inculation. These perturbations were counteracted by ellagic acid adminstration, indicating the protection against testicular dysfunction; findings that concurr with pioneering reports [11,18,30]. In fact, ACP and ALP activities are envisioned as functional indicators of spermatogenesis where ACP activity has been linked to testicular steroidogenesis. Increased ACP activity is also observed during germ cell differentiation from spermatogonia into spermatocytes and spermatids. Meanwhile, ALP plays a vital role in the process of spermatogenesis and has been shown to be vital for sperm survival and motility [31].

The current study revealed that adjuvant-induced arthritis lowered serum testosterone levels which is in accord with previous reports [3,7,17]. RA provokes damage to Leydig cells by induction of oxidative stress and suppression of steroidogenesis enzymes which are involved in testosterone synthesis [1]. Previous studies have indicated that men with RA have lower levels of serum testosterone than healthy men or male patients with osteoarthritis and ankylosing spondylitis [2]. The synthesis of testosterone (steroidogenesis) in Leydig cells is dependent on the expression of highly regulated genes such as StAR, 3β-HSD and 17β-HSD [32]. The observed suppression of testosterone production can be attributed to the accumulation of pro-inflammatory signals e.g., TNF-α which disturb Leydig cell function [33]. Additionally, the observed testicular expression of the pro-inflammatory COX-2 in arthritic rats is directly linked to the downregulation of StAR gene expression [32]. Likewise, increased oxidative stress markers in testicular tissues are also associated with suppressed StAR gene expression and steroidogenesis [10]. Interestingly, current results indicated that ellagic acid boosted testosterone in arthritic rats, mediated via upregulation of

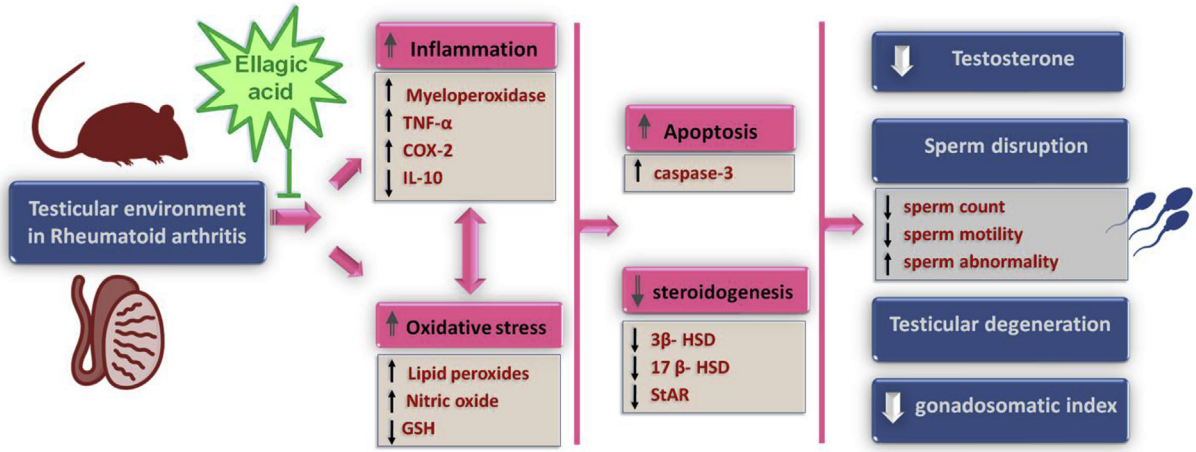

Fig. 10. A summary of the pathological changes that underlie rheumatoid arthritis-induced testicular impairment and the mitigation by ellagic acid. (→: activate; ┤: inhibit).

testicular steroidogenesis-associated 3β-HSD, 17β-HSD and StAR genes which coincide with earlier reports [10–12]. The observed boosting of testosterone production can be driven by ellagic acid-induced suppression of pro-inflammatory and pro-oxidant signals, culminating in the upregulation of StAR gene expression [10,17].

Inflammation is considered a crucial mechanism by which arthritis drives testicular injury [34]. In adjuvant arthritic rats, the present study revealed a severe testicular inflammation marked with increased expression of the pro-inflammatory MPO, TNF-α and COX-2 signals; findings that concur with earlier reports [3,17]. Pro-inflammatory cytokines e.g., TNF-α can halt androgen synthesis in the gonads by suppressing steroid 17 alpha-hydroxylase/17,20 lyase enzyme [35]. In addition, the observed upregulation of COX-2 expression can block Leydig cells steroidogenesis through downregulation of StAR gene [36]. Meanwhile, the observed lowering of the anti-inflammatory IL-10 exaggerates the testicular inflammation since IL-10 typically serves to suppress the expression of pro-inflammatory cytokines from activated neutrophils, macrophages and CD4 + T lymphocytes [17].

A distinctive finding of the current work is the efficacy of ellagic acid for suppressing paw inflammation as well as testicular impairment. This is consistent with the reported anti-inflammatory actions of ellagic acid in RA pathogenesis [16]. More important, the present data demonstrated that ellagic acid suppressed testicular inflammatory response via lowering of MPO, TNF-α and COX-2 levels and enhancing IL-10. Evolving evidence has pointed to the involvement of ellagic acid anti-inflammatory features for attenuation of several toxicant-induced testicular pathologies [10,11], concanavalin-induced hepatic injury [37] and dimethylhydrazine-provoked colon carcinogenesis in rats [38]. Of note, the observed ellagic acid boosting of testosterone has been reported to suppress RA inflammatory events. This is supported by the fact that boosting of serum testosterone can improve the clinical outcomes in RA patients thanks to the proven anti-inflammatory and immune-suppressive actions of testosterone [1,29]. In this context, testosterone has been reported to diminish pro-inflammatory cytokine production by macrophages and monocytes and halt the activation of the nuclear factor kappa B in human fibroblasts [1].

The present findings revealed that the progression of RA provoked exaggerated ROS generation and enhanced testicular oxidative stress as demonstrated by overshooting of lipid peroxides/nitric oxide along with exhaustion of reduced glutathione anti-oxidant defenses. These events are consistent with earlier studies [3,17].The oxidative perturbations correlate well with the impairment of spermatozoa functions [18], whereas the testicular anti-oxidant capacity is a major determinant of the male fertility [10]. In fact, free radical attack can lead to oxidative damage to the lipids, proteins, and nucleic acids of the spermatozoa with consequent disruption of sperm functions. The testicular tissue is abundant with polyunsaturated fatty acids and is endowed with increased metabolic rates which make it more vulnerable to the oxidative insult [39]. Ample evidence exists that exaggerated ROS generation weakens testicular junction proteins and incurs germ cell apoptosis [5]. The current data also demonstrated that exaggerated ROS generation and overshooting of pro-inflammatory signals have precipitated testicular apoptotic death as evidenced by upregulated caspase-3 expression in testes of arthritis rats. In fact, caspase-3, a protease-family enzyme, is an established marker of apoptosis that serves to execute DNA damage [40]. Several signals have reported to drive testicular apoptosis including excessive ROS, nitric oxide and pro-inflammatory cytokines [41].

Ellagic acid counteracted these oxidative/apoptotic insults and enhanced the anti-oxidant defenses, pointing to the notion that its anti-oxidant protective features, are at least partly, involved in the attenuation of RA-linked testicular impairment. These beneficial effects coincide with earlier studies that described the implication of ellagic acid anti-oxidant [11,12,18,30,42] as well as anti-apoptotic [12] actions for combating several toxicant-induced testicular pathologies. The marked anti-oxidant actions of ellagic acid can be atributed to the four phenolic hydroxyl groups and the two lactones, which enable it to scavenge H_2O_2 and superoxide anion radicals. Meanwhile, chelation of iron and enhancement of testicular anti-oxidant capacity are contributing factors in its anti-oxidant impact [10].

5. Conclusions

The present work features the efficacy of ellagic acid for attenuation of RA-evoked testicular dysfunction and impaired spermatogenesis. These beneficial effects were elicited via curbing the inflammatory, oxidative and apoptotic alterations. In fact, the current study represents a proof-of-concept framework that may support the role of ellagic acid as a safe adjunct agent for RA testicular impairment and disturbed spermatogenesis. Further studies are needed to delineate the detailed molecular signaling that mediated its beneficial actions.

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Author contributions

HHA, AMG, EMF and AHE conceived and designed the experiments.

HHA, AMG, EMF and AHE performed the experiments. HHA, AMG, EMF and AHE contributed reagents/materials/analysis tools. HHA, AMG, EMF and AHE analyzed the data. HHA, AMG, EMF and AHE wrote and approved the manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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