



Apigenin and baicalin, each alone or in low-dose combination, attenuated chloroquine induced male infertility in adult rats

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ABSTRACT

Introduction: Male infertility is a worldwide health problem, which accounts for about 50% of all cases of infertility and considered as the most common single defined cause of infertility. Recently, apigenin and baicalin exhibited a powerful antioxidant and antiapoptotic activities. Consequently, in the present study, we evaluated the possible protective effect of apigenin and baicalin, either alone or in low-dose combination, on a rat model of male infertility, regarding for its effects on the hormonal assay, testicular weight, sperm parameters, oxidative-stress state, apoptosis, and histopathological changes. **Material and methods:** 12-week-old adult male Wister rats received 10 mg/kg/d chloroquine orally for 30 days to induce male infertility. Either apigenin (30 or 15 mg/kg/d), baicalin (100 or 50 mg/kg/d) or a combination of 15 mg/kg/d apigenin and 50 mg/kg/d baicalin received daily orally 1 h after chloroquine for 30 days, used to protect against chloroquine induced male infertility. **Results and conclusion:** Our result showed that both apigenin and baicalin, significantly and dose-dependently enhanced the reduced levels of serum testosterone, follicular stimulating, and luteinizing hormones (LHs), testicular weight, reduced-glutathione (GSH) level, and catalase (CAT) activity, sperm count, mobility, and viability, and suppressed the elevated testicular malondialdehyde (MDA) level, and caspase-3 immunohistochemical expression, as well as they, abrogated the disturbed testicular histopathological pictures, induced by chloroquine, with superiority of baicalin. Furthermore, the low-dose combination therapy was as effective as the high dose apigenin therapy, except for sperm viability, where it was as powerful as the baicalin high dose. Therefore, both apigenin and baicalin either alone or in low-dose combination could be promising in the management of male infertility.

Keywords: Apigenin, apoptosis, baicalin, chloroquine, male infertility, oxidative stress

INTRODUCTION

Male infertility is a worldwide health problem, which accounts for about 50% of all cases of infertility, and considered as the most common single defined cause of infertility.^[1,2] It is defined by the World Health Organization as the male whose semen volume <1.5 ml, pH <7.2, sperm count <15 × 10⁶ spermatozoa/ml, motility below 40% forward progressive regardless the speed, morphology <40% of normal forms, and vitality below 58%.^[3] Many causes may induce male infertility, which may be pre-testicular, testicular, or post-testicular causes; however, more than 30% of cases remain idiopathic, and the exact cause is unknown. In these idiopathic cases, no

reliable medications are present, and researchers still search for the exact underlying pathogenesis, hence, the efficient treatment.^[2,4] Moreover, most of the available medications have many adverse effects, and with no obvious improvement in sperm parameters, and its successes are unpredictable.^[5]

Furthermore, except for the well-defined medical and surgical causes of male infertility, the underlying pathogenesis is complicated and involve complexing mechanisms. Of these mechanisms, reactive oxygen species (ROS) generation, hormonal disturbance, and apoptosis play a fundamental role in the development of male infertility.^[6-9] Therefore, we are in a great need to search for a new molecule, preferably of

plant origin, that could ameliorate these disorders, with the improvement of semen quality, hence curing male infertility.

Apigenin, 40,5,7-trihydroxyflavone, is a natural flavonoid abundantly presents in fruits and vegetables, such as apple, oranges, celery, and garlic as well as chamomile and Propolis.^[10] Recently, it exhibited antioxidant, antiapoptotic, anti-inflammatory, anticancer, anti-allergic, antimicrobial, neuroprotective, cardioprotective, and hormonal modulating activities; thus it could be considered as a health-promoting agent.^[11] Besides, it has an aromatase inhibiting property; hence, it could enhance the androgen level and hypothalamic-pituitary-gonadal axis, with the improvement of semen parameters.^[12] Moreover, it inhibits hepatic microsomal enzymes, cytochrome P450, thus, preventing other medications metabolism and excretion, with increasing of its bioavailability and therapeutic efficacy.^[13] From these powerful potentialities of apigenin, it could be a promising agent for treatment of male infertility alone or in combination with another effective agent.

Baicalin, syn-baicalin 7-O- β -D-glucuronic acid, is the main flavonoid presents in the roots of *Scutellaria baicalensis* Georgi, which used for centuries in the folk medicine as antimicrobial, anthelmintic and diuretic.^[14] At present, baicalin exerted a powerful antioxidant, antiapoptotic, anti-inflammatory, anti-allergic, antiplatelet, antimicrobial, anticancer, antidiabetic immunomodulating, hepatoprotective, cardioprotective, and photoprotective properties.^[15,16] Moreover, most recently, baicalin in a dose-dependent manner proved to be protective for testicular torsion-detorsion injury through its antioxidant, anti-inflammatory, and antiapoptotic activities.^[17] Therefore, in the present study, we evaluated the possible protective effect of apigenin and baicalin, either alone or in combination, on a rat model of testicular male infertility, regarding for its effects on the serum hormonal assay, testicular weight, sperm parameters, oxidative stress state, apoptosis, and histopathological changes.

MATERIALS AND METHODS

Drugs and Reagents

Apigenin and baicalin obtained from Sigma Co., St. Louis, Mo, USA. Moreover, chloroquine phosphate purchased from Pharaonia Pharma., New Borg El Arab, Alexandria, Egypt, and Bouin's solution, carboxymethylcellulose (CMC), phosphate buffered saline (PBS), xylol, citric acid, methanol, hydrogen peroxide, hematoxylin, and eosin stain from Al-Gomhoria pharmaceutical Co., Tanta, Egypt. In addition, biotinylated goat anti-polyvalent antibody, diaminobenzidine, and human serum albumin purchased from Lab Vision, Thermo Fisher Scientific Inc. Fremont, CA, USA. However, anti-caspase-3 polyclonal antibody obtained from Biocare Medical, Pacheco, CA, USA, Block Ace from DS Pharma Biomedical Co., Ltd., Osaka, Japan, pentobarbital sodium from Abbott Lab., Chicago, IL, USA, and normal saline 0.9% from Egypt Otsuka Co., 10th of Ramadan, Egypt.

Animals

The animals used in the present study were 12-week-old adult male Wister rats, weighing 150–200 g, and obtained

from Tanta University animal house, Tanta, Egypt. Rats were kept in meshed-plastic cages, at room temperature, with 12 h light/dark cycle, had free access to standard laboratory diet and water, and permitted to acclimatize for 1 week before the experimentation. All experiments carried out between 9:00 AM and 4:00 PM, after its approval by the Research Ethics Committee of Tanta Faculty of Medicine (Approval code 20160830), and followed its guideline for the care and use of experimental animals.

Experimental Design

Sixty-four rats randomly divided into 8 groups of 8 rats each. Group 1 (CON) was normal rats received 0.5 ml distilled water daily orally. Group 2 (CQ) was chloroquine induced male infertility group, which received orally chloroquine 10 mg/kg/d (dissolved in distilled water), for 30 days.^[18] Group 3 (CMC), male infertility induced group that treated with 0.5 ml 0.5% CMC daily orally. Group 4 (APH), male infertility induced group that treated orally with apigenin 30mg/kg/d (suspended in 0.5% CMC).^[19] Group 5 (APL), male infertility induced group that treated orally with apigenin 15 mg/kg/d (suspended in 0.5% CMC). Group 6 (BAH), male infertility induced group that treated orally with baicalin 100 mg/kg/d (suspended in 0.5% CMC).^[17] Group 7 (BAL), male infertility induced group that treated orally with baicalin 50 mg/kg/d (suspended in 0.5% CMC). Group 8 (AB), male infertility induced group that treated orally with apigenin 15 mg/kg/d (suspended in 0.5% CMC) and baicalin 50 mg/kg/d (suspended in 0.5% CMC). All treatments, freshly prepared daily, and delivered for 30 days, between 9:00 AM and 12:00 PM, 1 h after chloroquine receive.

Samples Collection

24 h after the last treatment, rats anesthetized with intraperitoneal injection of 50 mg/kg pentobarbital sodium. Then, blood collected through the cardiac puncture, centrifuged at 5000 rpm for 10 min, sera reaped and stored at -80°C for further hormonal assay. Afterward, animals sacrificed through cervical dislocation, dissected and both epididymis and testes harvested. Rapidly, both epididymis, weighed with PS-750.R1, RADWAG Wagi Elektroniczne, Poland, cut into small pieces, immersed in 37°C 5 ml 0.9% saline for 10 min, afterward, the sperm-containing fluid used for assessment of sperm parameters.^[20] Simultaneously, the right testis instantly processed for histopathological and immunohistochemical evaluation. Meanwhile, the left testis weighed with PS-750.R1, RADWAG Wagi Elektroniczne, Poland. Next, it's homogenized in 8 ml cold buffer, consist of 50 mmol potassium phosphate (pH 7.4), 1 mmol EDTA and 1 ml/l Triton X-100, for each tissue, then the homogenate divided into 2 equal parts, the first part centrifuged for 15 min at 4000 rpm, and the supernatant collected. The homogenate and the supernatant stored -80°C for further assessment of tissue oxidative stress markers.

Serum Hormonal Assay

For serum hormonal assay assessment, we measured serum testosterone (TE), follicle-stimulating hormone (FSH), and LH levels. Serum TE level evaluated according to manufacturer's

instruction of enzyme-linked immune-sorbent assay (ELISA) kit, obtained from Abcam, Cambridge, UK, with minimal detection limit 0.07 ng/ml. However, serum FSH and LH levels analyzed using ELISA kits purchased from Elabscience, Memorial Drive, Suite, Houston, Texas, USA, with minimal detection limits 1.88 ng/ml and 0.94 mIU/ml, respectively, according to producer's instructions. All absorbance analyzed with an automated ELISA plate reader (Stat Fax 2100, Fisher Bioblock Scientific, BR, Illkirch Cedex, France).

Sperm Parameters

Sperm parameters assessed according to the WHO laboratory manual for the examination and processing of semen.^[3]

Sperm Count

20 μ l of well-mixed seminal fluid placed under the glass cover of the Neubauer's hemocytometer chamber, and then sperm allowed settling down for 5 min, afterward, the number of sperm in the five small squares of Neubauer's hemocytometer chamber counted. The sperm count revealed by the following formula "total sperm count = number of sperm counted \times Dilution/number of squares \times 4" and expressed as million/ml.

Sperm Motility

Immediately after collection of semen, 10 μ l of well-mixed seminal fluid placed over a dry clean microscope slide and covered with a coverslip to make 20 μ m deep chamber, then wait for 60 s till sample stopped drifting, after that sample assessed under phase-contrast light microscope at \times 400, and the percentage of motile sperm, per 200 counted sperm, recorded. Each sample examined 3 times simultaneously, and the average expressed as a percentage of the total.

Sperm Viability

Directly after semen collection, 5 μ l of well-mixed seminal fluid added to 5 μ l of eosin stain over a dry clean microscope slide and mixed well with a pipette tip. Then, a coverslip placed over it and left for 30 s. Later, sperm viability evaluated under the negative phase contrast light microscope at \times 400, viable sperm has unstained or light pink head, while, the dead one has red or dark pink head, and the percentage of viable sperm, per 200 counted sperm, noted. Each sample examined 3 times simultaneously, and the average expressed as a percentage of the total.

Testicular Oxidative Stress Markers

Tissue MDA, reduced GSH levels, and CAT activity measured to assess testicular oxidative stress state. All tissue oxidative stress marker estimated with a spectrophotometric assay kit obtained from Bio-Diagnostic Co., Dokki, Giza, Egypt. Tissue MDA level evaluated according to the method that reported by Kei (1978). Briefly, 0.2 ml of homogenate' supernatant mixed well with 1 ml of 25 mmol/l thiobarbituric acid, heated in a water bath for 30 min (min) at 95°C, then allowed to cool. After that, the absorbance determined at 534 nm, and the MDA level expressed as nmol/g tissue.^[21] Moreover, tissue GSH level assessed according to Beutler *et al.* (1963). Concisely, 0.5 ml of tissue homogenate added to 0.5 ml

of 500 mmol/l trichloroacetic acid, mixed well and allowed to stand for 5 min. Then, the mixture centrifuged at 3000 rpm for 15 min, the supernatant removed and the aliquot collected. After that, the aliquot mix well with 0.1 ml of 1 mmol/l dithiobis-nitrobenzoic acid, and the absorbance evaluated at 412 nm after 5 min. The tissue GSH level expressed as μ mol/g tissue.^[22] However, the tissue CAT activity measured according to Aebi. Shortly, 0.05 ml of tissue homogenate' supernatant added to 0.5 ml of 100 mmol/l phosphate buffer (pH 7.0), and 0.1 ml of 1:1000 diluted 500 mmol/l hydrogen peroxide, incubated for 1 min, then, 0.5 ml of 2 mmol/l peroxidase 4-Aminoantipyrine added, incubated for 10 min at 37°C, and the absorbance assessed at 510 nm. The tissue CAT activity expressed as U/g tissue, where, one unit decomposes 1.0 μ mol of hydrogen peroxide per minute at pH 7.0.^[23] All absorbance determined using Biosystems semiautomatic analyzer (BTS-350, Barcelona, Spain).

Histopathological Examination

The right testis instantly processed according to Suvarna *et al.* It immediately immersed in Bouin's solution, embedded in paraffin and sectioned into 5 μ m sections.^[24]

Hematoxylin and Eosin (H and E) Stain

Sections deparaffinized in the oven with xylene, hydrated with a descending concentration of ethyl alcohol, stained with H and E stain, and examined under light microscope for histopathological changes. Moreover, the testicular section scored for histopathological changes according to Johnsen. In short, all tubular sections in one section scored into 10 grades as follow, 1 - no cells in tubular section, 2 - no germ cells, however, sertoli cells present, 3 - spermatogonia only present, 4 - <5 spermatocytes present, with no spermatozoa or spermatids, 5 - more than 5 spermatocytes present, with no spermatozoa or spermatids, 6 - <10 spermatids present, with no spermatozoa, 7 - more than 10 spermatids present, with no spermatozoa, 8 - <10 spermatozoa in the section, 9 - more than 10 spermatozoa in the section, with disorganized epithelium and obliterated lumen, and 10 - full spermatogenesis, with normal tubular structure.^[25]

Immunohistochemical caspase-3 expression

The method designated by Buchwalow and Böcker used for assessment of testicular immunohistochemical caspase-3 expression. In short, testicular sections deparaffinized with xylene and hydrated with descending concentration of ethyl alcohol, and then it incubated with 0.3% hydrogen peroxide/methanol for 20 min, for blocking of its endogenous hydrogen peroxidase activity. Next, it washed with PBS for 3 times and placed in 10 mmol citrate buffer solution (pH 6.0). Afterward, it incubated for 20 min at room temperature with Block Ace, a serum-free protein-blocking solution, for blocking of the non-specific protein binding sites, then, incubated with the anti-caspase-3 polyclonal antibody (diluted 1:100 in PBS) overnight at 4°C. Then, it washed with PBS for 3 times and incubated with secondary biotinylated goat anti-polyvalent antibodies for 10 min at room temperature. Subsequently, 2 drops of diaminobenzidine added to each section, counterstained with hematoxylin, dehydrated, mounted, and examined under a light microscope, where the positive reaction appeared brown,

while, the negative one was blue.^[26] Furthermore, the intensity of the testicular caspase-3 expression semi-quantified at $\times 200$ in 10 different regions for each section, and scored as follow, zero, no positive reaction detected, 1, <10% cells showed positive reaction, 2, 10 - 50% cells showed positive reaction, and 3, >50% cells showed positive reaction.

Statistical Analysis

Data of the present study expressed as a mean \pm standard error of the mean (SEM). The statistical difference between two groups detected either by the two-sample Student's *t*-test or the Mann-Whitney's *U*-test, after analysis of the variances by *F*-test. However, the statistical difference between multiple groups evaluated by either one-way ANOVA that followed by Tukey's test as a *post hoc* test, or Kruskal-Wallis's test that followed by Mann-Whitney's *U*-test as a *post hoc* test, after analysis of the variances by Bartlett's test. $P < 0.05$ considered significant.

RESULTS

Effect of Apigenin and/or Baicalin on Serum Hormonal Assay Levels

Chloroquine administration significantly reduced the serum TE, FSH, and LH levels, as compared with the control group. Both apigenin and baicalin treatment efficiently and dose-dependently enhanced the reduced level of serum TE, FSH, and LH levels, with the superiority of baicalin therapy. Moreover, the treatment with low-dose apigenin enhanced the hormonal elevating effect of the low-dose baicalin treatment, in the combination therapy, to be better than the effect of each alone. In addition, the low-dose combination therapy was better than the high dose apigenin treatment in elevating the

reduced hormonal level. However, baicalin high dose exerted the most prominent effect [Figure 1].

Effect of Apigenin and/or Baicalin on Testicular Weight

The testicular weight reduced significantly by administration of chloroquine when compared with the control group. The treatment with both apigenin and baicalin increased the reduced testicular weight in a dose-dependent manner. Moreover, the baicalin treatment showed an upper hand over the apigenin therapy in the elevation of the reduced testicular weight. In addition, baicalin low-dose, in the low-dose combination therapy, potentiated the normal testicular weight restoration of apigenin low-dose, to be as effective as apigenin high dose. Nevertheless, the baicalin high dose treatment was more effective in elevation of the reduced testicular weight than the low-dose combination therapy [Figure 2].

Effect of Apigenin and/or Baicalin on Sperm Parameters

Sperm count

Chloroquine administration significantly reduced the sperm count, as compared to the control group. The treatment by either apigenin or baicalin dose-dependently and effectively elevated the reduced sperm count, with the dominance of baicalin. Moreover, the low-dose combination therapy of both apigenin and baicalin was more effective than the low-dose apigenin, and as equal as to the effect of both high dose apigenin and low-dose baicalin; however, it was less effective than baicalin high dose, in restoration of normal sperm count [Figure 2].

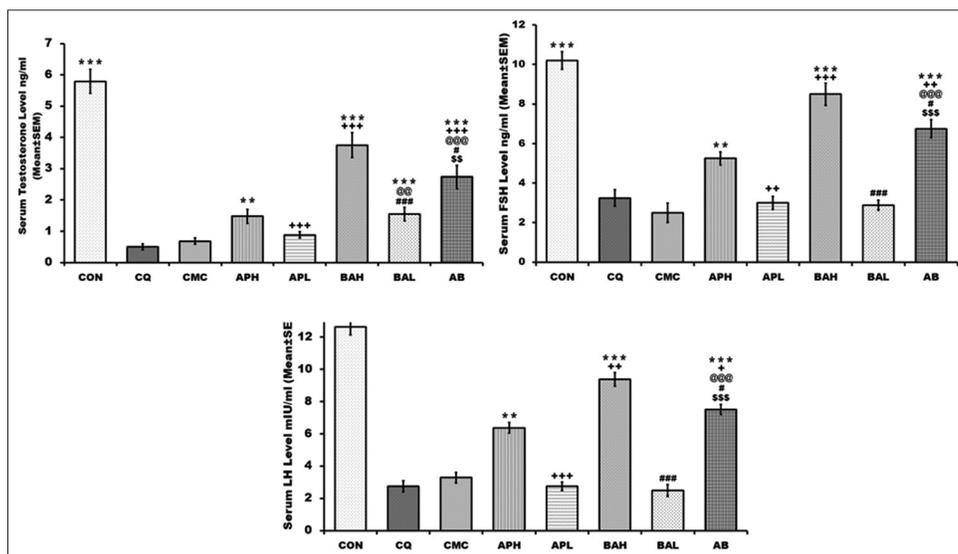


Figure 1: Effect of apigenin and/or baicalin on serum hormonal assay level of chloroquine induce male infertility in adult male Wister rats. Results expressed as mean \pm standard error of the mean of 8 rats per each group. CON, normal rats received 0.5 ml distilled water daily orally, CQ, chloroquine induced male infertility group, carboxymethylcellulose (CMC), male infertility induced group treated with 0.5 ml CMC 0.5% daily orally, APH, male infertility induced group treated orally with apigenin 30 mg/kg/d, APL, male infertility induced group treated orally with apigenin 15 mg/kg/d, BAH, male infertility induced group treated orally with baicalin 100 mg/kg/d, BAL, male infertility induced group treated orally with baicalin 50 mg/kg/d, AB, male infertility induced group treated orally with apigenin 15 mg/kg/d and baicalin 50 mg/kg/d. $***P < 0.01$ and $****P < 0.001$ (vs. CQ group), $+P < 0.05$, $++P < 0.01$ and $+++P < 0.001$ (vs. APH group), $@P < 0.01$ and $@@P < 0.001$ (vs. APL group), $#P < 0.05$ and $###P < 0.001$ (vs. BAH group), and $##P < 0.01$ and $###P < 0.001$ (vs. BAL group)

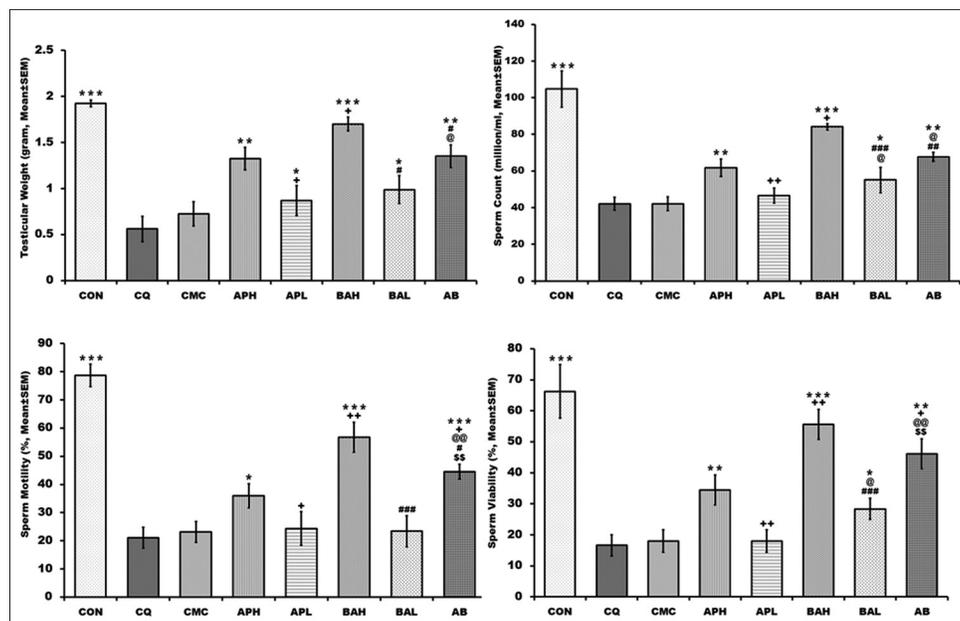


Figure 2: Effect of apigenin and/or baicalin on testicular weight and sperms parameters of chloroquine induce male infertility in adult male Wister rats. Results expressed as mean \pm standard error of the mean of 8 rats per each group. CON, normal rats received 0.5 ml distilled water daily orally, CQ, chloroquine induced male infertility group, carboxy-methylcellulose (CMC), male infertility induced group treated with 0.5 ml CMC 0.5% daily orally, APH, male infertility induced group treated orally with apigenin 30 mg/kg/d, APL, male infertility induced group treated orally with apigenin 15 mg/kg/d, BAH, male infertility induced group treated orally with baicalin 100 mg/kg/d, BAL, male infertility induced group treated orally with baicalin 50 mg/kg/d, AB, male infertility induced group treated orally with apigenin 15 mg/kg/d, and baicalin 50 mg/kg/d. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (vs. CQ group), + $P < 0.05$ and ++ $P < 0.01$ (vs. APH group), @ $P < 0.05$ and @@ $P < 0.01$ (vs. APL group), # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ (vs. BAH group), and \$\$ $P < 0.01$ (vs. BAL group)

Sperm motility

The apigenin and baicalin treatment efficiently enhanced the suppressed sperm motility, induced by chloroquine, in a dose-dependent manner. Moreover, the high dose baicalin therapy is more effective than the apigenin high dose in the enhancement of sperm motility. In addition, the sperm motility enhanced with the low-dose combination therapy to be more efficient than apigenin high and low doses, and baicalin low dose, though, it was less effective than the baicalin high dose therapy [Figure 2].

Sperm viability

Both the apigenin and baicalin dose-dependently and significantly promoted the reduced percentage of sperm viability induced by chloroquine administration. Yet, baicalin therapy was superior to the apigenin therapy, in the enhancement of sperm viability. Furthermore, the combination of low-dose apigenin and low-dose baicalin therapy was more significant than apigenin high and low doses, and baicalin low dose in the improvement of sperm viability. Nevertheless, it was as significant as baicalin high dose in the promotion of sperm viability [Figure 2].

Effect of Apigenin and/or Baicalin on Testicular Oxidative Stress Markers

The administration of chloroquine disturbed the testicular antioxidant state significantly, which evidenced by the increase of testicular MDA level and the reduction of testicular GSH level and CAT activity. However, the treatment with

both apigenin and baicalin dose-dependently recovered the disturbed antioxidant state, which revealed by the reduction of the elevated testicular MDA level, as well as, the elevation of the reduced testicular GSH level and CAT activity, with the superiority of baicalin. Moreover, in the combination therapy, the low-dose apigenin enhanced the antioxidant activity of low-dose baicalin, to be more effective than each alone, except for the MDA level, where it was as effective as the low-dose baicalin therapy. Similarly, the low-dose combination therapy was as efficient as the high dose apigenin therapy, except for the improvement of CAT activity. Yet, the baicalin high dose therapy showed an upper hand over the low-dose combination therapy as an antioxidant [Figure 3].

Effect of Apigenin and/or Baicalin on Testicular Histopathological Changes

H and E sections

The control group H and E sections exhibited normal testicular structure, with oval or rounded seminiferous tubules that lined with a thick stratified germinal epithelium resting on a basement membrane. Moreover, its stratified germinal epithelium composed of two types of cells, spermatogenic cells at various stages of maturation, and supporting Sertoli cells. The spermatogenic cells arranged in 5–6 layers between the basal lamina and lumen of the tubules that appeared as small round cells. Besides, spermatids were arranged in two to three rows close to the lumen of the tubules. Furthermore, the tubular lumens filled with elongated mature spermatozoa. Nevertheless, the CQ, CMC, and APL groups'

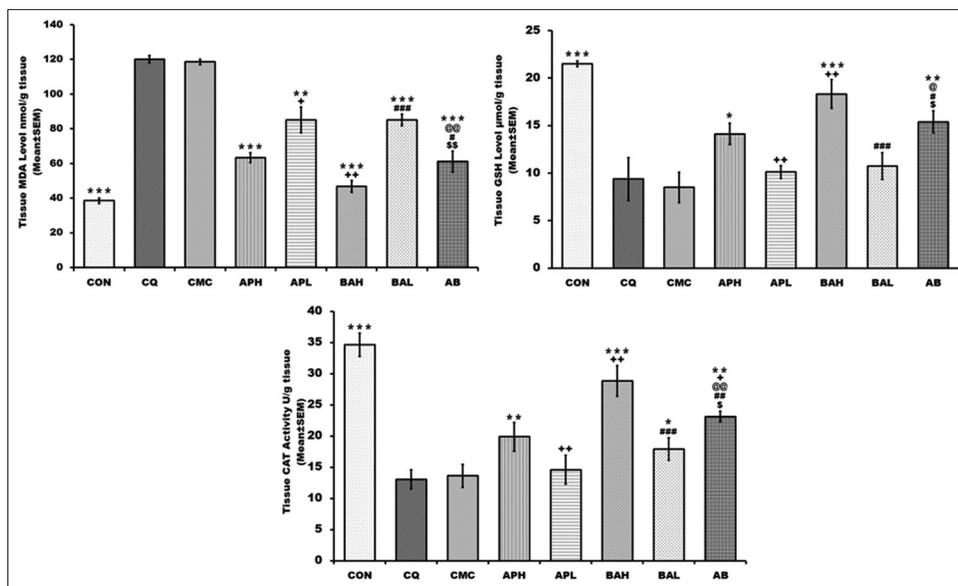


Figure 3: Effect of apigenin and/or baicalin on testicular oxidative stress markers of chloroquine induce male infertility in adult male Wister rats. Results expressed as mean \pm standard error of the mean of 8 rats per each group. CON, normal rats received 0.5 ml distilled water daily orally, CQ, chloroquine induced male infertility group, carboxymethylcellulose (CMC), male infertility induced group treated with 0.5 ml CMC 0.5% daily orally, APH, male infertility induced group treated orally with apigenin 30 mg/kg/d, APL, male infertility induced group treated orally with apigenin 15 mg/kg/d, BAH, male infertility induced group treated orally with baicalin 100 mg/kg/d, BAL, male infertility induced group treated orally with baicalin 50 mg/kg/d, AB, male infertility induced group treated orally with apigenin 15 mg/kg/d, and baicalin 50 mg/kg/d. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (vs. CQ group), + $P < 0.05$ and ++ $P < 0.01$ (vs. APH group), @ $P < 0.05$ and @@ $P < 0.01$ (vs. APL group), # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ (vs. BAH group) and \$ $P < 0.05$ and \$\$ $P < 0.01$ (vs. BAL group)

sections exhibited a severely disturbed testicular structure as revealed by irregular outlines of seminiferous tubules, with severe degeneration and atrophy of spermatocytes that looked vacuolated, and sometimes ghost-like. Moreover, there was arrested spermatogenesis, with few widely separated spermatogonia, loss of spermatids and sometimes sloughed hyalinized necrotic sperm seen. In addition, the interstitial tissue showed severe congestion and hyalinized degeneration. Yet, the APH and ABL groups' section showed apparently mild restoration of the normal pattern of seminiferous tubules. As, few sections exhibited an arrested spermatogenesis at the level of spermatid, but most of the sections showed all stages of spermatogenesis; furthermore, the lumen of the seminiferous tubules contained few mature sperms. However, some spermatogenic cells and sperm showed hydropic degenerations, with vacuolation and degeneration. Besides, the interstitial edema and congestion apparently diminished. Furthermore, the BAH and AB groups' sections revealed a histopathological picture that was apparently more or less like the control group [Figure 4].

Histopathological scoring

Chloroquine administration induced severe histopathological changes in testicular tissue, as compared to the control group. However, the treatment with apigenin and baicalin efficiently and dose-dependently restored the normal histological structure of testis, with the superiority of baicalin. Moreover, the baicalin low-dose adds to the effect of the apigenin low dose to be as effective as apigenin high dose. Furthermore, the baicalin high dose therapy was the most effective in abrogation of the testicular histopathological changes induced by chloroquine [Figure 5].

Immunohistochemical caspase-3 expression

CQ and CMC groups exhibited a strong positive caspase-3 reaction, as compared to the negative reaction of the control group. However, the treatment with either apigenin or baicalin reduced effectively the immunohistochemical caspase-3 expression in a dose-dependent manner, with the dominance of baicalin. Moreover, the baicalin low-dose augments the effect of apigenin low dose to be as efficient as apigenin high dose. In addition, the baicalin high dose exerted the best reduction of caspase-3 immunohistochemical expression [Figures 5 and 6].

DISCUSSION

In the present study, we assessed the protective effect of apigenin and baicalin, either alone or in low-dose combination, on a rat model of male infertility, regarding for its effects on the serum hormonal assay, testicular weight, sperm parameters, oxidative stress state, apoptosis, and histopathological changes. Our finding showed that both apigenin and baicalin significantly and dose-dependently restored the normal serum hormonal assay, as revealed by the elevation of the reduced levels of TE, FSH, and LH, enhanced the diminished testicular weight, sperm count, mobility, and viability, recovered the normal testicular antioxidant state, as evidenced by the reduction of the increase testicular MDA level and raised the decreased testicular GSH level and CAT activity, and suppressed testicular immunohistochemical caspase-3 expression, induced by chloroquine. In parallel with these activities, both apigenin and baicalin therapy effectively abrogated the disturbed testicular histopathological pictures, induced by chloroquine, in a dose-dependent manner. Moreover, baicalin was dominant over apigenin in all of these abilities. Furthermore, the

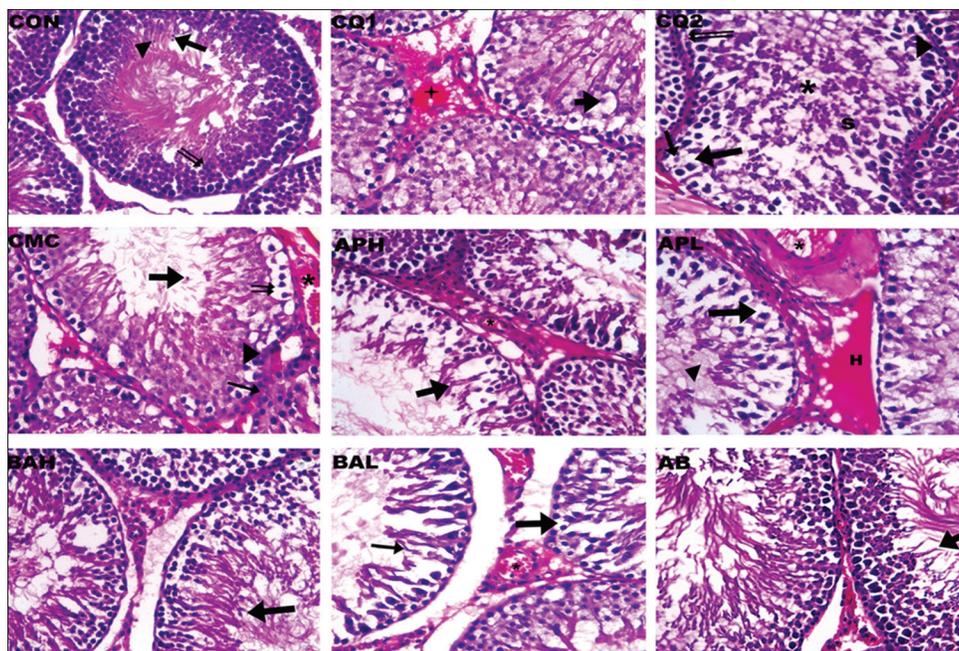


Figure 4: Effect of apigenin and/or baicalin on testicular histopathological changes of chloroquine induce male infertility in adult male Wister rats. CON, spermatogenic cells (double arrows), spermatids (thick arrow) and mature spermatozoa (▶), CQ1 and 2, spermatocytes filled with vacuoles (thick arrow), severe congestion of the interstitial tissue (star), irregular outlines seminiferous tubules (▶), ghost like spermatozoa (thin arrow), widely separated spermatogonia (double arrow), disarrangement of spermatids (S) and nearly absent of spermatozoa (*), carboxymethylcellulose, sloughed sperms (thick arrow), spermatocytes filled with vacuoles (double arrow), severe congestion of the interstitial tissue (*), irregular outlines seminiferous tubules (▶) and widely separated spermatogonia (thin arrow), APH, mild congestion of the interstitial tissue (*) and mature sperms (arrow), APL, marked interstitial congestion (*), interstitial hyalinized degeneration (H), vacuolated spermatocytes (arrow) and hyalinized necrotic sperms (▶), BAH, mature sperms (arrow), BAL, vacuolated spermatocytes (thick arrow), diminished sperm density (thin arrow) and mild congestion of the interstitial tissue (*) and AB, mature sperms (arrow)

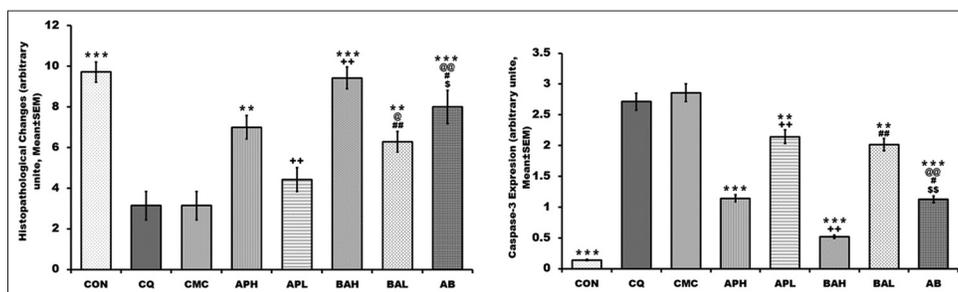


Figure 5: Effect of apigenin and/or baicalin on testicular histopathological scoring and immunohistochemical caspase-3 expression of chloroquine induce male infertility in adult male Wister rats. Results expressed as a mean ± standard error of the mean of 8 rats per each group. CON, normal rats received 0.5 ml distilled water daily orally, CQ, chloroquine induced male infertility group, carboxymethylcellulose, male infertility induced group treated with 0.5 ml CMC 0.5% daily orally, APH, male infertility induced group treated orally with apigenin 30 mg/kg/d, APL, male infertility induced group treated orally with apigenin 15 mg/kg/d, BAH, male infertility induced group treated orally with baicalin 100 mg/kg/d, BAL, male infertility induced group treated orally with baicalin 50 mg/kg/d, AB, male infertility induced group treated orally with apigenin 15 mg/kg/d, and baicalin 50 mg/kg/d. ***P* < 0.01 and ****P* < 0.001 (vs. CQ group), ++ *P* < 0.01 (vs. APH group), @ *P* < 0.05 and @@ *P* < 0.01 (vs. APL group), # *P* < 0.05 and ## *P* < 0.01 (vs. BAH group) and \$ *P* < 0.05 and \$\$ *P* < 0.01 (vs. BAL group)

low-dose apigenin, in the combination therapy, promoted the low-dose baicalin to be as efficient as the high dose apigenin therapy in all of these capabilities, except for sperm viability, where it was as powerful as the baicalin high dose therapy.

Although many animal models have been developed for assessment of the underlying pathogenesis of male infertility, and to evaluate the efficacy of new agents for its management, we selected the model of testicular atrophy induced by chloroquine in rats, to explicit male infertility, in the present

study. As, chloroquine induced testicular atrophy in rats that match the fundamentals features of male infertility in human, including disturbance in FSH, LH, and TE hormones levels, reduction of testicular weight, sperm count, motility and viability, as well as, disruption of testicular antioxidant activity, in addition to its exhibition of similar apoptotic and histopathological changes.^[18,27-30]

Apart from the well-known etiologies of male infertility, nearly about 30% are still idiopathic, with no clear

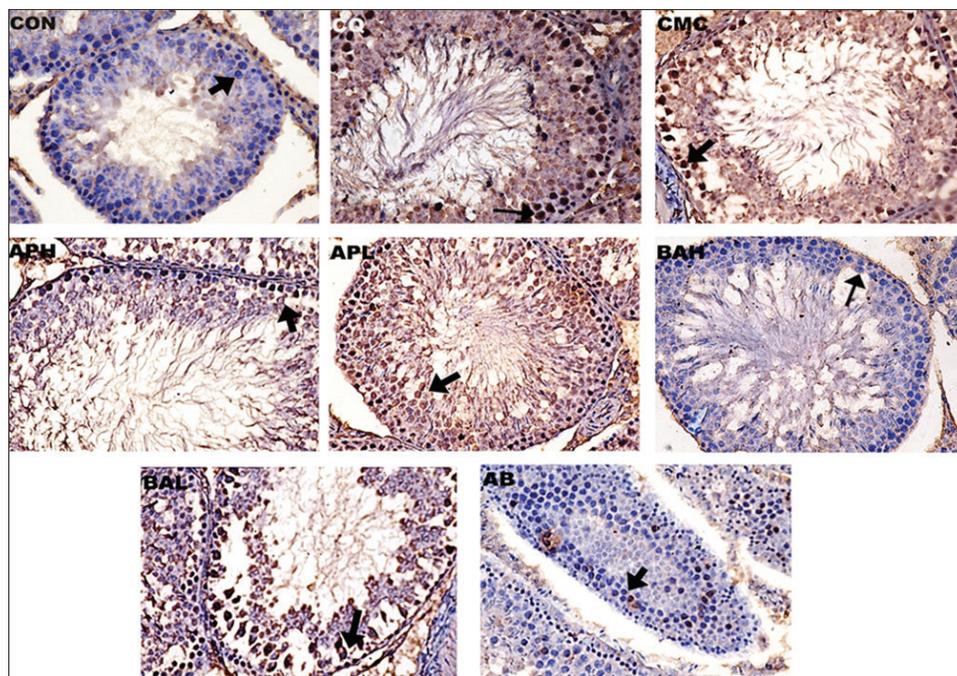


Figure 6: Effect of apigenin and/or baicalin on testicular immunohistochemical caspase-3 expression of chloroquine induce male infertility in adult male Wister rats. CON, negative caspase-3 immunoreactivity (blue cytoplasm, →), CQ, strong positive caspase-3 immunoreactivity (brown cytoplasm, →), carboxymethylcellulose, strong positive caspase-3 immunoreactivity (brown cytoplasm, →), APH, mild positive caspase-3 immunoreactivity (→), APL, moderately positive caspase-3 immunoreactivity (→), BAH, nearly negative caspase-3 immunoreactivity (→), BAL, mild positive caspase-3 immunoreactivity (→), AB, nearly negative caspase-3 immunoreactivity (→)

pathogenesis that could clarify these conditions.^[2] Moreover, the underlying pathogenesis of male infertility is complicated and involves complexing mechanisms.^[12] One of the crucial mechanisms that involve in the development of male infertility is ROS generation, which believed to account for about 30–80% of male infertility as a major contributor.^[6,8] Although, the presence of low level of ROS in sperm is essential for its maturation and fertilization reactions, however, if it exceeds the ability of the sperm's antioxidant system, it becomes a destructive to sperm. As, the sperm cell membrane contains a high level of polyunsaturated fatty acids, which are vulnerable to ROS, moreover, it oxidized sperm proteins, lipids, and DNA, as well as it impairs mitochondrial function and ATP production, resulting in sperm dysfunction and damage, with an ultimate infertility.^[8,31] Furthermore, semen of infertile men displayed a higher level of ROS, which correlates inversely with their sperm motility and viability, fertilization, pregnancy outcomes, and offspring's deformities.^[31-33] In addition, ROS induce DNA fragmentation, with a subsequent viable DNA absence or transmission of damaged DNA that hinder fertilization, affecting pregnancy outcomes or ends with offspring deformities, also, ROS-induced DNA fragmentation initiates the germ cells apoptosis cascade, resulting in decline of sperm count and viability, hence, male infertility.^[34] Meanwhile, ROS promote Leydig cells aging and apoptosis, which consequently decreased testosterone, LH stimulated cAMP and androgen binding protein production, thus decreased libido and sperm dysfunction.^[35] Thus, agents with ROS scavenging activity could help in the restoration of fertility for men whose semen produces high levels of ROS and/or has the low ROS scavenging capacity.^[8]

In the present study, chloroquine administration disturbed the testicular antioxidant state, as displayed by diminishing of testicular GSH level and CAT activity, meanwhile, elevation of the testicular MDA level. Matching with our finding, chloroquine reported to enhance ROS generation through suppression of enzymatic and non-enzymatic antioxidant activity.^[28,36] In addition, chloroquine reduced irreversibly sperm count, motility, and viability, *in vitro*, through ROS generation, hence, inhibition of DNA synthesis and repair, as well as induction of DNA intercalation.^[28,37] In contrary, both apigenin and baicalin, in the present study, exerted a powerful antioxidant activity in a dose-dependent manner. In parallel with our data, apigenin reduced acrylonitrile induced sub-chronic sperm injury and heat-induced testicular damage in rats and mice, respectively, through enhancement of testicular superoxide dismutase and GSH peroxidase activity, and decreasing of testicular MDA level.^[38,39] Further, apigenin expressed an efficient cardioprotective power through elevation of cardiac GSH level, superoxide dismutase, and CAT activity, along with declining in MDA level.^[40] In addition, it attenuated the bleomycin-induced pulmonary fibrosis, through increasing lung levels of GSH and superoxide dismutase.^[41] On the other hand, baicalin protected against testicular torsion-detorsion injury in rats, by reduction of testicular MDA and nitric oxide levels, and promotion of testicular GSH peroxidase and superoxide dismutase activities.^[17] In addition, baicalin suppressed oxidative damage of testes in heat stressed mice, through the diminishing of testicular MDA level, and endorsement of testicular superoxide dismutase, GSH peroxidase, and CAT activities.^[42] Therefore, low-dose combination of both apigenin and baicalin potentiate the

antioxidant activists for each other to be as efficient as the high dose apigenin therapy.

Another fundamental mechanism for the development of male infertility is disruption of the hypothalamic–pituitary–gonadal axis. As, hypothalamus stimulates FSH and LH release from the anterior pituitary gland, that, in turn, stimulate germinal epithelium and Leydig cells to produce sperm and testosterone, respectively.^[12] In addition, the released TE is important in the initiation and maintenance of spermatogenesis, through completion of the meiotic division and spermatid development. In addition, TE exerts negative feedback on the hypothalamus and anterior pituitary by inhibin B and directly by itself.^[7] Thus, interruption of this axis leads to inhibition of spermatogenesis, with consequent male infertility. As well, it was reported that nearly 50% of all men with high ROS idiopathic male infertility suffer from hypogonadotropic hypogonadism.^[43]

Our finding disclosed that chloroquine administration, diminished serum TE, FSH, and LH levels, illuminating the disturbance of the hypothalamic-pituitary-gonadal axis, accordingly it repressed spermatogenesis that could be elucidated by reduction of sperm count and testicular weight. Coherent with our data, chloroquine administration proved to reduce serum TE level, so hindering spermatogenesis, and the secondary sexual characters development and maintenance, with consequent decreasing of sexual abilities in rats. Moreover, it decreases testicular volume up to 64% of control, with a reduction in seminiferous tubule length and diameter, and disruption of the normal histological structure of rat's testis.^[28,29] However, both apigenin and baicalin dose-dependently elevated the reduced serum levels of TE, FSH, and LH, with the restoration of normal sperm count and testicular weight. Matching with these data, apigenin enhanced the serum TE level in heat-induced testicular damage in mice.^[39] Further, *Carissa opaca* leaves and *Matricaria chamomilla* extract, where apigenin is one of their fundamental bioactive ingredients, elevated the reduced serum levels of TE, FSH, and LH in carbon tetrachloride-induced reproductive stress in male rats, and in the neonatal period of rats, respectively.^[44,45] Likewise, apigenin is a powerful inhibitor of the aromatase enzyme; consequently, it reduces plasma estradiol level, with subsequent stimulation of hypothalamic–pituitary–gonadal axis that increases TE, FSH, and LH levels, and promotes spermatogenesis.^[46] Opposite to our finding, Hui *et al.* (2010) reported that apigenin could be injurious to the adult male mice reproductive system, with suppression of the germ cells proliferation speed, and blocking of spermatogonia in cell-circle phase G0/G1, thus decreased sperm density. This controversy could be due to use of different animal species, apigenin dose, route, and period of administration.^[47] In addition, although baicalin exerts, in previous studies, an anti-androgenic activity on human dermal papilla cells and human sebocytes, our data showed that baicalin effectively raised the reduced serum level of TE, which could be through the protection of Ledge cells by its antioxidant and antiapoptotic activities.^[48,49] In addition, the joining of antioxidant, antiapoptotic, and hepatic cytochrome P450, and aromatase inhibiting activities could explain why the combination of both low-dose apigenin and low-dose baicalin was as powerful as high dose apigenin in the enhancement of TE, FSH, and LH levels.

Furthermore, apoptosis is another essential mechanism for the development of male infertility. Actually, apoptosis plays an important role in the maintenance of male fertility through maintaining the normal ratio between germ cells and Sertoli cells, and the appropriate number of premeiotic spermatogonia as well as removal of defective germ cells or sperm.^[50] Although the increased rate of germ cell apoptosis, through ROS generation and DNA fragmentation, account for the reduction of sperm count and viability, therefore male infertility, which could be revealed by high levels of caspase activity.^[51]

Our finding revealed that chloroquine administration, enhanced caspase-3 immunohistochemical expression, consequently, it induced apoptosis with reduction of sperm count and viability and testicular weight. In accordance with our data, chloroquine decreased the rate of fertilization and the number of embryos of cohabited female rat, by lessening of sperm motility and viability through induction of sperm apoptosis.^[18,27] Moreover, it decreases testicular volume up to 64% of control, with a reduction in seminiferous tubule length and diameter, and disruption of the normal histological structure of testis.^[28,29] However, both apigenin and baicalin dose-dependently suppressed caspase-3 immunohistochemical expression, clarifying apoptosis inhibition. In parallel with our results, Apigenin protected against ischemia-reperfusion and heat-induced testicular damage, and acrylonitrile induced sub-chronic sperm injury, in rats' and mice's testes, through its effective antiapoptotic activity.^[38,39,52] In addition, baicalin suppressed apoptosis in testicular torsion-detorsion injury in rats that demonstrated by suppression of caspase-3 expression, thus protected the testes against it.^[17] Furthermore, baicalin prevented heat stress-induced apoptosis in Sertoli cells by activation of the Fas/FasL pathway and Hsp72 expression.^[53] Likewise, baicalin protected against heat-induced mice testicular damage, through its powerful antiapoptotic activity.^[42] In the light of apigenin and baicalin's combined antiapoptotic activities, it could explicate why the combination of the low dose apigenin and low dose baicalin, was as competent as high dose apigenin in restoration of sperm count and motility, and testicular weight. In addition, it was as powerful as high dose baicalin in restoration of sperm viability and in abrogation of the chloroquine induced testicular histopathological changes.

In addition, apigenin is a potent inhibitor of cytochrome P450, chiefly CYP1A1, CYP1A2, and CYP3A4.^[13] Moreover, CYP3A4 is the main cytochrome P450 enzyme responsible for the metabolism of baicalin, which proved that its inhibition increases the circulating level of baicalin, hence its biliary excretion.^[16] Taking into consideration the previous data, we could explain the ability of apigenin, in the low-dose combination therapy, to potentiate baicalin's antioxidant, antiapoptotic, and hormonal modulating activities, thus augmentation of its fertility preserving potentiality. In addition, chloroquine mainly metabolized by cytochrome P450 CYP1A1, CYP2D6, CYP3A4, and CYP2C8,^[54,55] where baicalin has no effect on their activities.^[56] However, apigenin is a potent inhibitor of CYP3A4, thus could maintain the circulating level of chloroquine and its bioavailability.^[13] These data could elucidate the superiority of baicalin over apigenin in conservancy of male fertility, although of the potent

antioxidant, antiapoptotic, and aromatase inhibiting activities of apigenin.

CONCLUSION

Both apigenin and baicalin, either alone or in low-dose combination, able to protect against chloroquine induced male infertility in a dose-dependent manner, through their antioxidant and antiapoptotic activities as well as their abilities to restore the normal hypothalamic–pituitary–gonadal hormonal balance, with the superiority of baicalin. Therefore, both apigenin and baicalin either alone or in low-dose combination could be hopeful agents in the male infertility management.

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