ISSN 0120-4157

Biomédica

Revista del Instituto Nacional de Salud

PUBLICACIÓN ANTICIPADA EN LINEA

El Comité Editorial de *Biomédica* ya aprobó para publicación este manuscrito, teniendo en cuenta los conceptos de los pares académicos que lo evaluaron. Se publica anticipadamente en versión pdf en forma provisional con base en la última versión electrónica del manuscrito pero sin que aún haya sido diagramado ni se le haya hecho la corrección de estilo.

Siéntase libre de descargar, usar, distribuir y citar esta versión preliminar tal y como lo indicamos pero, por favor, recuerde que la versión impresa final y en formato pdf pueden ser diferentes.

Citación provisional:

Jazayeri O, Araghi SF, Aghajanzadeh TA, Mohammadrezaei FM. Up-regulation of Arl4a gene expression by broccoli aqueous extract is associated with improved spermatogenesis in mouse testes. Biomédica. 2021;41 (4).

Recibido: 21-01-21

Aceptado: 15-07-21

Publicación en línea: 27-07-21

Up-regulation of *Arl4a* gene expression by broccoli aqueous extract is associated with improved spermatogenesis in mouse testes

La regulación positiva de la expresión del gen Arl4a por acción del extracto acuoso de brócoli se asocia con una mejor espermatogénesis en los testículos de ratón

***Brassica oleracea* extract improves spermatogenesis**

Omid Jazayeri [1], Setareh Farahmand Araghi [1], Tahereh A. Aghajanzadeh [2], Fereshteh Mir Mohammadrezaei [3]

[1] Department of Molecular and Cell Biology, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

[2] Department of Plant Science, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

[3] Department of Animal Science, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

Corresponding Author:

Omid Jazayeri, Department of Molecular and Cell Biology, Faculty of Sciences, University of Mazandaran, Babolsar, Iran.

Mobile: +989111143017; fax: +98-11-35302450

ojazayeri@gmail.com; o.jazayeri@umz.ac.ir

Author contributions:

Tahereh Aghajanzadeh and Omid Jazayeri are responsible for the concept, design of experiments, practical experiments, interpretation of the results and supervision of the study.

Setareh Farhmand Araghi performed the experiments in the laboratory and also was involved in writing the manuscript.

Fereshteh Mir Mohammadrezaei helped with animal experiments and performance and interpretation of the sperm factors results.

Omid Jazayeri wrote the manuscript.

Introduction: Broccoli (*Brassica oleracea*) is well recognized due to its properties as an anti-cancer, antioxidant and scavenging free radicals. However, its benefit in enhancing spermatogenesis is not well understood.

Objectives: To investigate the effect of broccoli aqueous extract on sperm factors and also expression of the involving genes (*Catsper1*, *Catsper2*, *Arl4a*, *Sox5* and *Sox9)* in sperm factors in mice.

Material and methods: Male mice were divided randomly into six groups: (1) Control, (2) Cadmium (3 mg/kg mouse body weight), (3) Orally treated with 200 broccoli aqueous extract (1 g ml^{-1}), (4) Orally treated with 400 µl of broccoli aqueous extract, (5) Orally treated with 200 broccoli aqueous extract plus cadmium, and (6) Orally treated with 400 µl of broccoli aqueous extract plus cadmium. Sperms factors and also gene expression in *Catsper1*, *Catsper2*, *Arl4a*, *Sox5* and *Sox9* genes were studied.

Results: An obvious improvement in sperm number and slight enhancement in sperm motility was observed in mice treated with broccoli extract with and without cadmium. While sperm viability was reduced by broccoli extract, except for 200 µl of broccoli extract with cadmium that was significantly increased. Interestingly, *Arl4a* gene expression showed an increase in 400 µl broccoli-treated group. Likewise, the *Arl4a* mRNA level in mice treated with cadmium along with 200 µl broccoli extract was higher than in cadmium-treated mice. Furthermore, broccoli extract enhanced the mRNA level of *Catsper2* and *Sox5* genes in mice treated with both 200 and 400 µl broccoli extract along with cadmium than the only cadmium-treated group.

Conclusion: Generally, improvement in sperm count in broccoli-treated mice provides insight into the pharmaceutical industry to make new products available to infertile men.

Keywords: Brassica; spermatogenesis; gene expression; mice; cadmium.

Palabras clave: Brassica; espermatogénesis; expression génica; ratones; cadmio.

Infertility is defined by failure to achieve the clinical pregnancy after one year or more of regular unprotected sexual intercourse. Of all infertility cases, approximately 40–50% is due to male factor (1). Developing reproductive technologies can conquer male infertility; however, the genetic defects may still be passed to the male's offspring (2). Medicinal plants are approaches to discover new drugs and health care as well. For instance, Trigonellae Semen, which is derived from *Trigonella foenum-graecum* L., is commonly used as a medicinal herb for the treatment of infertility in Korean medicine (2). Additionally, *Panax ginseng* is a traditional medicinal plant that is applied for male infertility. Rats treated with ginseng had a significant increase in sperm count and sperm motility (3,4). *Aspalathus linearis* and *Camellia sinensis* have also a forceful positive impact on sperm factors (5). Decursin extracted from *Angelica gigas* has also shown a positive effect on sperm counts and motility in cryptorchidism-induced infertile rats (6). The protective effect of *Crocus sativus* L. on cadmium (Cd) toxicity in rat spermatogenesis has been reported, too (7). Recent evidence suggests that the Ca^{2+} levels of mice sperm cells treated with *P. ginseng* extract increases significantly compared with the normal group and induces the expression of *Catsper* genes (the gene involved in sperm motility and fertility) (8,9).

There is a growing body of literature on therapeuetic effect of cruciferous plants like broccoli, which has been attributed largely to their high content of glucosinolates. Broccoli is well recognized worldwide as an anti-cancer medicinal plant (10), antioxidant as the finest natural active substance for scavenging free radicals (11,12), antigenotoxic agent (13), reducer of fasting blood glucose in type 2 diabetes patients (14), and act in protection of cardiovascular (15), central nervous system, diabetic nephropathy and

neuropathy (16), restoration of skin integrity (17), protection against *Helicobacter pylori* infection (18), and improvement in social interaction in patients with autism (19).

Heavy metal pollutants like Cd and lead are considered as sources of significant environmental damage. Indeed, they do not have any biological functions and can be extremely toxic even at low concentrations (20). The toxic effect of heavy metals and their health hazards in living organisms are comprised of various diseases such as cancer, infertility, nephritis, hair loss, brain damage, cardiovascular disease, low blood pressure and paralysis. However, kidney and lung damage, fragile bones, change in calcium regulation in biological systems has been recognized as a specific toxicological influence of Cd (21).

The effect of broccoli on spermatogenesis and their related gene expression in mouse testes has yet to be determined. The genetic bases and molecular mechanisms underlying spermatogenesis and its molecular regulation are not fully understood. Therefore, in the current study, the impact of broccoli extract on sperm count and gene expression of *Arl4a* - the gene involved in spermatogenesis (22), the gene expression of *Catsper1* and *Catsper2* - the genes involved in sperm motility (23); as well as genes expression of *Sox5* and *Sox9* - transcription factors which are involved in Sertoli cells and sex development have been investigated (24).

Material and method

Experimental animals

This study was conducted after being approved by the Ethics Committee of the University of Mazandaran, Iran (IR.UMZ.REC.1399.002). NMRI male mice (5–6 weeks old mouse with the weight between 25-30 g) were purchased from the Pasteur Institute

(Tehran, Iran). Then, the mice were kept for one week in polycarbonate cages for adaptation to the new environment to reach the desired conditions. The animals were kept under standard conditions of the environment (12 h light/dark cycle at 24 ± 2 °C) with free access to drinking water and standard pellets. Once every two days, the cages were cleaned and the remaining food was collected and again fresh food was available to the mice. Mice were anesthetized intraperitoneally with ketamine hydrochloride (100 mg kg^{-1}) and xylazine (5 mg kg^{-1}).

Preparation of broccoli aqueous extract

The two-month broccoli was collected from a local greenhouse and immediately was frozen and transferred to the laboratory of plant physiology. The broccoli flowers were grinned in liquid nitrogen to a fine powder, dissolved in boiling water and boiled for 3 min (1 g ml^{-1}). Then, the suspension was filtered through one layer of filter paper and the extraction was centrifuged at 10,000g for 10 min at 4°C and stored at −20°C for further experiments (25).

Experimental design

Thirty-six NMRI male mice were divided randomly into six groups with six mice in each group: Control, (2) cadmium (3 mg/kg mouse body weight), (3) Orally treated with 200 broccoli aqueous extract (1 g ml^{-1}), (4) Orally treated with 400 µl of broccoli aqueous extract, (5) Orally treated with 200 broccoli aqueous extract plus cadmium, and (6) Orally treated with 400 µl of broccoli aqueous extract plus cadmium. The mice in the control group were only received distill water. In the Cd group, mice were injected intraperitoneally with only cadmium chloride (3 mg/kg mouse body weight). Four different groups were administered by oral gavage at 200 and 400 µl of BE (1 g ml^{-1}) /60

g of mouse body weight with and without intraperitoneally injection of cadmium chloride. The BE was applied every day for 48 days and Cd on the last day of BE treatment. On the 49th day of the experiment, all animals were sacrificed and samples were collected. The sperms rapidly were detached from the epididymis and were used to determine the parameters. The testis from each mouse separated and immediately frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

Evaluation of cauda epididymal sperm count and motility

Seminal plasma aspirated from the caudal part of the epididymis in mice was used for a semen analysis. The epididymis was put in a solution containing 1 mL of PBS buffer (pH = 7) and then was cut by razor to small pieces. Tissue homogenate was incubated at 37°C for 10 min for releasing sperm in the solution. Ten µl of the sperm was placed on a clean slide coverslip for the evaluation of sperm count and motility (26). Total sperm counts were determined with a haemocytometer under a light microscope at 200× magnification. Spermatozoa were collected with a micropipette to a slide for motility estimation and were classified into motile and non-motile sperms. Sperm motility was expressed as the percentage of sperm that showed any movement (fast forward, slow movements and move in place) and data were expressed as percentages (26).

Sperm viability

Viability was assessed by eosin-nigrosin solution (26). A 10µl sample of the sperm suspension was placed on a glass slide, mixed with 10 µl eosin; after drying at laboratory temperature observed under a light microscope (400x magnification). The head of the spermatozoa absorbs eosin and becomes red, but live spermatozoa remain colorless due to the failure of the plasma membrane. Live spermatozoa were counted

from each sample in five fields of vision randomly, and the percentage of live spermatozoa was recorded.

Semi-quantitative RT-PCR

RNA extraction and cDNA synthesis

Total RNA was extracted from the testis using RNX plus (EX6101) solution according to the manufacturer's protocol (SINACLON, Iran). The RNA pellet was dissolved in RNase-free water (DEPC treated water). Total RNA was then treated with DNAaseI (SINACLON, Iran) to destroy possible genomic DNA contamination, followed by heat treatment (65°C for 10 minutes) to inactivate the enzyme and then stored at − 80 °C for the future cDNA synthesis. The purity and integrity of the extracted total RNA were checked, by 260/280 nm ratio using Thermo Scientific NanoDrop spectrophotometer and visualized on 1% agarose gel. A commercial cDNA synthesis kit (2-steps RT-PCR kit, vivantis, Malaysia) was used and the recommended procedures of the manufacturer were utilized. Briefly, 1 µg of total RNA, 0.5 µl M-MuLV Reverse Transcriptase (200 U/ µl), 2 µl of 10X Buffer M-MuLV, 1 µl Oligo d(T)18 (40µM), 1 µl of 10mM dNTPs were added to a 0.2 ml microcentrifuge tube. The reaction mixture adjusted to the final volume 20 µl by nuclease-free water.

Polymerase Chain Reaction (PCR)

Primer sequences, GeneBank ID and amplification product sizes are summarized in table 1. Primers were synthesized by Metabion (Germany). Samples were normalized with the *β actin* gene as a reference gene for its constitutive expression. Synthesized cDNA was amplified by PCR reaction. PCR was performed by using 2x PCRBIO Taq Mix Red in a reaction volume of 12.5 µl containing: 6.25 µl 2x PCRBIO Taq Mix Red 0.5

µl of each of forward and reverse primers (10 pM), 1.5 µl cDNA and 3.75 µl H2O. PCR conditions for *Catsper1*, *Catsper2* and *Sox9* were: 94°C for 2 min followed by 36 cycles (94°C for 30 s, 57°C for 30 s and 72°C for 1 min as an extension), and a final extension at 72°C for 10 min. For *Sox5* was: 94°C for 2 min followed by 32 cycles (94°C for 30 s, 60 °C for 30 s and 72°C for 1 min as an extension), and a final extension at 72°C for 10 min. For *Arl4a* and *β actin* were: 94°C for 2 min followed by 30 cycles (94°C for 30 s, 57°C for 30 s and 72°C for 1 min as an extension), and a final extension at 72°C for 10 min.

The number of PCR cycles for each gene was determined to obtain a detectable signal without reaching saturation. The electrophoresis of amplified products was carried out using 1% agarose gel. The amplified cDNA fragment was visualized and photographed under UV light. The band intensities were semi-quantitatively analyzed using E-capt Software (Vilber Lourmat, France) and normalized against that of *β actin*. The obtained data were expressed as the mean and standard deviation (SD) of at least 3 PCR replicates.

GeneMANIA in silico analysis to study protein-protein interaction

We utilized a large set of protein-protein interaction databases within the GeneMANIA package (30) to build a protein-protein interaction network. This package comprises 244 databases/articles that collectively utilize experimentally proved physical protein-protein interactions in human. Arl4a was applied as input for the GeneMANIA algorithm. Although we used “mice” in this investigation, we selected “Homo sapiens” as organism in GeneMANIA input setting, because protein-protein interaction in “human” is more

complete than “mouse”. Thus, this choice provides more insight into Arl4a biological functions.

Statistical analysis

All data were analyzed by Prism software using one way ANOVA and Tukey post-test at a significant level of $p \leq 0.05$.

Results

Impact of broccoli extract on body and testis weight with and without Cd-toxicity

As it was shown in Table 2, no significant difference was evident between groups in both body weight and testis weight.

Impact of broccoli extract on sperm parameters with and without Cd-toxicity

Figure 1 displays microscopic images of sperm in NMRI mice treated with Cd and BE. Figure 2 provides experimental data on sperm parameters. In Figure 2A, a positive significant impact of BE on sperm count has been observed and was in a dose-dependent manner. The sperm count in the BE-treated (400µl) group was 1.5 and 2-fold higher than those in broccoli extract-treated (200µl) group and control one, respectively (Figure 1 A, C,E). In addition, a clear benefit of BE in the prevention of Cd-toxicity could be identified on sperm count. Likewise, the sperm count was significantly increased more in BE-treated groups (both 200 and 400µl) along with Cd than the mice only treated with Cd almost 2.5 and 2.7-fold (see Figures 1 B, D, F).

Furthermore, sperm viability was clearly reduced in Cd-treated mice compared with the control group (figure 1 A, B and figure 2B). When Cd-treated mice were in 200 and 400 µl (1g/ml) broccoli extract, the sperm viability was enhanced by 5.3 and 1.5-fold, respectively (Figure 2B).

However, sperm viability was hardly changed in mice treated with 200 µl BE and even extremely reduced in mice treated with 400 µl BE (Figure 2B). Additionally, sperm motility was significantly decreased in Cd-treated mice (figure 2C) and their treatment with both 200 and 400 µl BE led to an increase in sperm motility. Likewise, the mice treated with both doses of BE exhibited higher sperm motility than in mice in the control group.

Impact of broccoli extract on gene expression of Catsper1, Catsper2 and Arl4a with and without Cd-toxicity

Figure 3 shows the relative gene expression by semi-quantitative RT-PCR. What stands out in Figure 4 is that no significant difference among groups was evident related to *Catsper1* gene expression.

The gene expression of *Catsper2* in Cd-treated mice was significantly lower than in the other groups (Figure 4). However, there was not significant difference among BE-treated groups with and without Cd-toxicity, and a closer look of the graph indicated that the gene expression of *Catsper2* in both groups treated with 200 and 400 µl BE with Cd were significantly higher than in the mice treated only with Cd (Figure 4).

The mRNA level of the *Arl4a* gene was up-regulated (1.8-fold) in the group treated with 400 µl BE (figure 4). Furthermore, it was clearly observed that *Arl4a* gene expression was down-regulated in the group treated only with Cd. However, the gene expression of *Arl4a* in both groups treated with 200 and 400 µl BE along with Cd was higher than in the mice treated only with Cd (figure 4).

Impact of broccoli extract on gene expression of transcription factors SOX5 and SOX9 with and without Cd-toxicity

Figure 5 provided the experimental data with gene expression of two transcription factors (*Sox5* and *Sox9*). Following the intraperitoneal administration of Cd, a significant decrease in the *Sox5* gene expression was identified. However, no significant differences in gene expression of *Sox5* were found between BE-treated mice groups (200 and 400 µl BE) with the control group, the mice treated with BE together with Cd significantly up-regulated compared to the mice treated with the only Cd. Furthermore, the gene expression of *Sox9* appeared to be unaffected by Cd. Likewise, none of the BE-treated mice groups with and without Cd treatment showed a significant differences in *Sox9* mRNA level (figure 5).

Discussion

Currently, only few publications have so far been reported related to the association between spermatogenesis and BE (31,32). The present study was designed to determine the effect of BE on sperm factors as well as some genes expression in which their relation has already been reported for spermatogenesis. The most obvious and interesting finding emerging from figure 2A is the number of sperms that was significantly increased in BE-treated groups in a dose-dependent manner. Even in the presence of Cd, the extract could increase the number of sperms. What is surprising is that *Arl4a* gene expression also showed an almost similar increase in sperm count in both BE-treated groups with and without Cd. ARL4 is a 22-kDa GTP-binding protein which is abundant in testes of pubertal and adult rodents. Mouse *Arl4*-null mutants (*Arl4*$^{-/-}$) indicated that inactivation of the *Arl4* gene causes a significant reduction of testis weight and sperm count by 30 and 60%, respectively (22). Furthermore, an association between testis weight and the *Arl4a* gene has been reported in the literature (33).

Genome-wide mapping in the house mouse revealed a significant association between SNPs located in the *Arl4a* gene and relative testis weight. However, no significant difference between body and testis weight among six investigated groups was found in this study. Similarly, Zhou et al did not note significant effects on body and testis weight when they treated mice with broccoli seed extract (0.3, 1 and 3 g/kg body weigh/day) for 30 days (34), and the finding shows that the LD_{50} of broccoli seed extract in rats was >10 g/kg body weight/day.

Even though the role of the *Arl4a* gene in mouse spermatogenesis has been discovered in 2002 (22), very little is currently known about Arl4a biological functions and its mechanism related to spermatogenesis. Therefore, we tried to find more supportive evidence about Arl4a and its role in spermatogenesis. *In silico* analysis of Arl4a and the proteins that are able to bind it physically by the GeneMANIA algorithm could expand our knowledge. As it was shown in Figure 6, Arl4a was bound to Spatc1l through the protein-protein interaction. It was previously indicated that Spatc1l maintains the integrity of the sperm head-tail junction and Spatc1l knockout mouse undergoes male sterility owing to the separation of sperm heads from tails (35). Kpna2 also presented the protein-protein interaction with Arl4a. The literature review revealed that it is a key mediator of nucleocytoplasmic transport in the embryonic testis. *Kpna2* mRNA was identified in both pachytene spermatocytes and round spermatids (36) and the amount of Kpna2 highly elevates within spermatocytes and spermatids (37). Golga2 another protein that physically binds to Arl4a plays a role in spermatogenesis. Han and colleagues showed that the inactivation of *Golga2* caused male infertility in the mouse model. *Golga2*$^{-/-}$ mouse model exhibited the absence of acrosome and round sperm

heads which are characteristic features of human globozoospermia (38). Likewise, Cytoskeleton was disorganized in *Golga2*$^{-/-}$ testes. During spermatogenesis, the ubiquitin–proteasome pathway (UPP) plays a key role in this process, which facilitates the formation of condensed sperm, and the implemented UPP blocks spermatogenesis. Ubiquitination occurs in different cell types during spermatogenesis, especially in spermatocyte, round spermatid, and elongating spermatid even mature sperm in the epididymis (39). As it was shown in Figure 4, ubiquitin C (UBC), one of the ubiquitination enzymes, can also physically bind to Arl4a. Inadequate studies have focused on the association between CCDC102B and spermatogenesis. This protein functions in centrosome linker assembly and requires for maintaining centrosome cohesion. It provides forces that hold the duplicated centrosomes together and prevents centrosome separation. The regulation of connection and/or disconnection of two centrosomes is involved in several cellular processes such as Golgi and cilia positioning (40). CCDC57, Coiled-Coil Domain Containing protein 57, is not also a well-documented protein functionally; however, it may play a role in centrosome, as it possesses coiled-coil domain (41).

Interestingly, Arl4 was found to have two separate promoters in the rat. Jacobs and colleagues revealed that mRNA transcription is under the control of the downstream promoter in most tissues while the upstream promoter seems to drive specifically the expression of Arl4 in adult testis (42). In fact, they recorded tissue-specific alternative splicing and alternative promoter use in the *Arl4* gene. These findings provide insights into the molecular control of Arl4a protein in spermatogenesis. Apparently, Arl4a have

various biological functions in different tissues, different organelles and its expression is regulated developmentally.

Broccoli can accumulate selenium and consequently has been demonstrated to reduce the risk of several cancers (43,44). Additionally, selenium is essentially required for spermatogenesis and male fertility (45). Treating with selenium significantly increased the gene expression of both C*atsper1* and *2* in adult male mice (46); therefore, it is able to enhance sperm mobility. The results of our study indicated that *Catsper2* was also up-regulated in dose-dependent BE; however, the *Catsper1* mRNA level did not display a significant difference. We also observed an obvious increase in spermatogenesis by BE which is consistent with the selenium enrichment of broccoli and its positive impact on spermatogenesis. Our finding is in accord with Raeeszadeha et al. who observed number of spermatogonium, primary spermatocytes, spermatids and sperm count were significantly increased by hydroalcoholic extract of broccoli (300 mg/kg) in male mice after 42 days of treatment (32).

Consistent with the literature, this research found that Cd-treated mice also displayed a reduction in sperm count, motility and viability as well as down-regulation of the *Catsper2* gene (47,48). However, the findings of the current study do not support Mohammadi and colleagues who observed a reduction of *Catsper1* mRNA level in Cd-treated mice (48). Of course, there is a complexity in *Catsper1* gene expression, *Catsper1* promoter is bidirectional and regulates the expression of a lncRNA (*Catsper1au*). Indeed, *Catsper1au* is expressed in adult male mouse testis and can regulate gene expression during spermatogenesis (49). It is possible that broccoli contains compounds that bind directly or indirectly to regulatory proteins that regulate

this lncRNA or binds to the *Catsper1au* target site or some other unknown phenomenon involved in *Catsper1* gene regulation.

Previous research has established that both Sox5 and Sox9 transcription factors interact with the *Catsper1* promoter in HEK-293 cells (50). As can be seen from Figure 5, the gene expression in *Sox9* was similar to the *Catsper1* gene and no differences observed among treatments in both of them, which is in accord with Mata-Rocha and colleagues' (2014) findings. However, our findings indicated that *Sox5* gene expression was reduced dramatically in Cd-treated mice than in the control. A deeper look revealed that in both 200 and 400 µl BE- treated mice *Sox5* mRNA level was slightly higher than Cd plus 200 and 400 µl BE-treated mice. This contrary behavior may be attributed to the other transcription factors or miRNA e.g. miR-195 which target Sox5 3' UTR and impairs its expression (51).

Quercetin and kaempferol are the predominant flavonoids in commercial broccoli (52). Quercetin attenuates Cd-induced oxidative damage and apoptosis in granulosa cells from chicken ovarian follicles (53). In spite of the antioxidant properties of quercetin, a significant concentration-dependent conflicting effect of quercetin on sperm viability and motility has already been observed (54,55) which is somewhat in line with our finding. Indeed, we observed that the broccoli diet affects positively on sperm count; however, it negatively impacts on sperm viability and also increased sperm motility slightly (figure 2). Raeeszadeh et al. determined that the total antioxidant IC_{50} of hydroalcoholic extract of broccoli is 278±14 µg/ml (32). Their finding probably explains the negative impact of broccoli extract on sperm viability and highlights the complexity of its antioxidant properties. Additionally, it is reported that treatment with quercetin markedly inhibited

nickel-induced global hyper-methylation and DNA hypo-methylation of the nuclear factor E2-related factor 2 (Nrf2) promoter (56) which may subsequently affect its downstream target genes. Kaempferol also inhibits DNA methylation by suppressing DNA methyltransferases (57). It has been previously observed that kaempferol leads to the downregulation of DNMT3 (a kind of DNA methyltransferase) and modulates DNA methylation in cancers (58). Indeed, kaempferol alters 103 DNA methylation positions (hypo-methylation and hyper-methylation) associated with bladder cancer genes (57). Likewise, sulforaphane, another predominant ingredient in broccoli increased *Nrf2* expression, a transcription factor, in prostate tumor cells through epigenetic regulation in mice (59). By activation of Nrf2/ARE signaling pathways, sulforaphane prevented testicular damage in Cd-treated mice (60). Sulforaphane also reduced Cd-induced toxic effects on human mesenchymal stem cells and showed a significant recovery of the cell viability (61). Dspite supprotive impacts of sulforaphane as an anticancer agent, the fact is that sulforaphane also interferes T cell-mediated immune response (62) and maybe a double-edged sword.

Growing body of literature reports on epigenetic regulation of broccoli ingredients and also a stable and reversible epigenetic mechanism in regulating gene expression. Therefore, further research needs to determine which compound(s) of broccoli extract stimulate(s) spermatogenesis without a negative effect on sperm viability. Studying a lower dosage of broccoli extract would be another opportunity to find a suitable concentration of broccoli without its negative effect on sperm viability and use it as a medicinal plant for male infertility. To have more precise view of gene expression alterations, performing the experiments by real-time PCR is suggested in future studies.

Homo sapiens-based instead of Mus musculus-based protein-protein interaction probably is not able to reflect exactly what happens in mouse; however, this *in silico* analysis definitely would be informative and supports Arl4a role in a spermatogenesis process. The implication of this study is that the pharmaceutical industry can benefit from this finding to produce medicine curing infertile men.

Funding

The project was supported by a grant from the University of Mazandaran.

Conflicts of interest

The authors declare that they have no conflict of interest.

Reference:

1. Kumar N, Singh AK. Trends of male factor infertility, an important cause of infertility: A review of literature. J Human Reprod Sci. 2015;8:191-6. https://doi.org/10.4103/0974-1208.170370
2. Kim DR, Kim HY, Kim HY, Chang MS, Park SK. Trigonellae semen enhances sperm motility and the expression of the cation sperm channel proteins in mouse testes. Evid Based Complementary Altern Med. 2015 ;2015:817324. https://doi.org/10.1155/2015/817324
3. Park WS, Shin DY, Yang WM, Chang MS, Park SK. Korean ginseng induces spermatogenesis in rats through the activation of cAMP-responsive element modulator (CREM). Fertil Steril. 2007;88:1000-2. https://doi.org/10.1016/j.fertnstert.2006.12.014
4. Yang WM, Park SY, Kim HM, Park EH, Park SK, Chang MS. Effects of Panax ginseng on glial cell-derived neurotrophic factor (GDNF) expression and

spermatogenesis in rats. Phytother Res. 2011;25:308-11. https://doi.org/10.1002/ptr.3239

5. Awoniyi DO, Aboua YG, Marnewick J, Brooks N. The effects of rooibos (Aspalathus linearis), green tea (Camellia sinensis) and commercial rooibos and green tea supplements on epididymal sperm in oxidative stress-induced rats. Phytother Res. 2012;26:1231-9. https://doi.org/10.1002/ptr.3717
6. Bae WJ, Ha U, Choi JB, Kim KS, Kim SJ, Cho HJ, *et al*. Protective effect of decursin extracted from Angelica gigas in male infertility via Nrf2/HO-1 signaling pathway. Oxid Med Cell Longev. 2016;2016:5901098. https://doi.org/10.1155/2016/5901098
7. Yari A, Sarveazad A, Asadi E, Raouf Sarshoori J, Babahajian A, Amini N, *et al*. Efficacy of Crocus sativus L. on reduction of cadmium-induced toxicity on spermatogenesis in adult rats. Andrologia. 2016;48:1244-52. https://doi.org/10.1111/and.12568
8. Park EH, Do Rim Kim HY, Park SK, Chang MS. Panax ginseng induces the expression of CatSper genes and sperm hyperactivation. Asian J Androl. 2014;16:845. https://doi.org/10.4103/1008-682X.129129
9. Sun XH, Zhu YY, Wang L, Liu HL, Ling Y, Li ZL, *et al*. The Catsper channel and its roles in male fertility: a systematic review. Reprod Biol Endocrinol. 2017;15:1-2. https://doi.org/10.1186/s12958-017-0281-2
10. Kensler TW, Egner PA, Agyeman AS, Visvanathan K, Groopman JD, Chen JG, *et al*. Keap1–nrf2 signaling: a target for cancer prevention by sulforaphane. Top Curr Chem. 2013; 329:163-77. https://doi.org/ 10.1007/128_2012_339

11. Yang SH, Yu LH, Li L, Guo Y, Zhang Y, Long M, *et al*. Protective mechanism of sulforaphane on cadmium-induced sertoli cell injury in mice testis via Nrf2/ARE signaling pathway. Molecules. 2018;23:1774. https://doi.org/10.3390/molecules23071774
12. Fahey JW, Wehage SL, Holtzclaw WD, Kensler TW, Egner PA, Shapiro TA, *et al*. Protection of humans by plant glucosinolates: efficiency of conversion of glucosinolates to isothiocyanates by the gastrointestinal microflora. Cancer Pre Res. 2012;5:603-11. https://doi.org/10.1158/1940-6207.CAPR-11-0538
13. López-Romero D, Izquierdo-Vega JA, Morales-González JA, Madrigal-Bujaidar E, Chamorro-Cevallos G, Sánchez-Gutiérrez M, *et al*. Evidence of some natural products with antigenotoxic effects. Part 2: plants, vegetables, and natural resin. Nutrients. 2018;10:1954. https://doi.org/10.3390/nu10121954
14. Conzatti A, da Silva Fróes FC, Perry ID, de Souza CG. Clinical and molecular evidence of the consumption of broccoli, glucoraphanin and sulforaphane in humans. Nutr Hosp. 2015;31:559-69. https://doi.org/10.3305/nh.2015.31.2.7685
15. Senanayake GV, Banigesh A, Wu L, Lee P, Juurlink BH. The dietary phase 2 protein inducer sulforaphane can normalize the kidney epigenome and improve blood pressure in hypertensive rats. Am J Hypertens. 2012;25:229-35. https://doi.org/10.1038/ajh.2011.200
16. Dinkova-Kostova AT, Kostov RV. Glucosinolates and isothiocyanates in health and disease. Trends Mol Med. 2012;18:337-47. https://doi.org/10.1016/j.molmed.2012.04.003

17. Kerns ML, DePianto D, Dinkova-Kostova AT, Talalay P, Coulombe PA. Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex.). Proc Natl Acad Sci. 2007;104:14460-5. https://doi.org/10.1073/pnas.0706486104

18. Yanaka A, Fahey JW, Fukumoto A, Nakayama M, Inoue S, Zhang S, *et al*. Dietary sulforaphane-rich broccoli sprouts reduce colonization and attenuate gastritis in Helicobacter pylori–infected mice and humans. Cancer Pre Res. 2009;2:353-60. https://doi.org/10.1158/1940-6207.CAPR-08-0192

19. Singh K, Connors SL, Macklin EA, Smith KD, Fahey JW, Talalay P, *et al*. Sulforaphane treatment of autism spectrum disorder (ASD). Proc Natl Acad Sci. 2014;111:15550-5. https://doi.org/10.1073/pnas.1416940111

20. Duruibe JO, Ogwuegbu MO, Egwurugwu JN. Heavy metal pollution and human biotoxic effects. Int J Phys Sci. 2007;2:112-8. https://doi.org/10.5897/IJPS.9000289

21. Mishra S, Bharagava RN, More N, Yadav A, Zainith S, Mani S, *et al*. Heavy metal contamination: an alarming threat to environment and human health. In: Sobti R, Arora N, Kothari R, editors. Environmental biotechnology: For sustainable future. Singapore: Springer, 2019. p. 103-25. https://doi.org/10.1007/978-981-10-7284-0_5

22. Schürmann A, Koling S, Jacobs S, Saftig P, Krauss S, Wennemuth G, *et al*. Reduced sperm count and normal fertility in male mice with targeted disruption of the ADP-ribosylation factor-like 4 (Arl4) gene. Mol Cell Biol. 2002;22:2761-8. https://doi.org/10.1128/MCB.22.8.2761-2768.2002

23. Pereira R, Sá R, Barros A, Sousa M. Major regulatory mechanisms involved in sperm motility. Asian J Androl. 2017;19:5. https://doi.org/10.4103/1008-

682X.167716

24. She ZY, Yang WX. SOX family transcription factors involved in diverse cellular events during development. Eur J Cell Biol. 2015;94:547-63. https://doi.org/10.1016/j.ejcb.2015.08.002
25. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci USA. 1997;94:10367-72. https://doi.org/10.1073/pnas.94.19.10367
26. World Health Organization. WHO laboratory manual for the examination and processing of human semen. Fifth edition. Switzerland: WHO Press; 2010. p. 286.
27. Mohammadi S, Movahedin M, Mowla SJ. Up-regulation of CatSper genes family by selenium. Reprod Biol Endocrinol. 2009;7:126. https://doi.org/10.1186/1477-7827-7-126
28. Suzuki N, Mizuniwa C, Ishii K, Nakagawa Y, Tsuji K, Muneta T, et al. Teneurin-4, a transmembrane protein, is a novel regulator that suppresses chondrogenic differentiation. J Orthop Res. 2014;32:915–22. https://doi.org/10.1002/jor.22616
29. Shiota M, Heike T, Haruyama M, Baba S, Tsuchiya A, Fujino H, et al. Isolation and characterization of bone marrow-derived mesenchymal progenitor cells with myogenic and neuronal properties. Exp Cell Res. 2007;313:1008-23. https://doi.org/10.1016/j.yexcr.2006.12.017
30. Zuberi K, Franz M, Rodriguez H, Montojo J, Lopes CT, Bader GD, *et al*. GeneMANIA prediction server 2013 update. Nucleic acids Res. 2013;41:W115-22. https://doi.org/10.1093/nar/gkt533

31. Khademi N, Raeeszadeh M, Allahveisi A. Effect of different concentrations of hydroalcoholic extract of broccoli on sperm parameters and oxidative stress factors before and after thawing in NMRI mice. J Shahid Sadoughi Univ Med Sci. 2019;26:921-33. http://dx.doi.org/10.18502/ssu.v26i10.482

32. Raeeszadeh M, Khademi N, Akbari A. The effects of broccoli and caraway extracts on serum oxidative markers, testicular structure and function, and sperm quality before and after sperm cryopreservation. Cryobiology. 2021;99:11-9. https://doi.org/10.1016/j.cryobiol.2021.02.003

33. Turner LM, Harr B. Genome-wide mapping in a house mouse hybrid zone reveals hybrid sterility loci and Dobzhansky-Muller interactions. eLife. 2014;3:e02504. https://doi.org/10.7554/eLife.02504

34. Zhou Y, Yang H, Li Y, Lynch B, Jia X. Broccoli seed extract: Genotoxicity and subchronic toxicity studies. Regul Toxicol Pharm. 2015;73:442–51. https://doi.org/10.1016/j.yrtph.2015.08.003

35. Kim J, Kwon JT, Jeong J, Kim J, Hong SH, Kim J, *et al*. SPATC 1L maintains the integrity of the sperm head-tail junction. EMBO Rep. 2018;19:e45991. https://doi.org/10.15252/embr.201845991

36. Hogarth CA, Calanni S, Jans DA, Loveland KL. Importin α mRNAs have distinct expression profiles during spermatogenesis. Dev Dyn. 2006;235:253-62. https://doi.org/10.1002/dvdy.20569

37. Major AT, Whiley PA, Loveland KL. Expression of nucleocytoplasmic transport machinery: clues to regulation of spermatogenic development. Biochim Biophys Acta Mol Cell Res. 2011;1813:1668-88.

https://doi.org/10.1016/j.bbamcr.2011.03.008

38. Han F, Liu C, Zhang L, Chen M, Zhou Y, Qin Y, *et al*. Globozoospermia and lack of acrosome formation in GM130-deficient mice. Cell Death Dis. 2018;8:e2532. https://doi.org/10.1038/cddis.2016.414
39. Hou CC, Yang WX. New insights to the ubiquitin–proteasome pathway (UPP) mechanism during spermatogenesis. Mol Biol Rep. 2013;40:3213-30. https://doi.org/10.1007/s11033-012-2397-y
40. Xia Y, Huang N, Chen Z, Li F, Fan G, Ma D, *et al*. CCDC102B functions in centrosome linker assembly and centrosome cohesion. J Cell Sci. 2018;131:jcs222901. https://doi.org/10.1242/jcs.222901
41. Nogales-Cadenas R, Abascal F, Díez-Pérez J, Carazo JM, Pascual-Montano A. CentrosomeDB: a human centrosomal proteins database. Nucleic acids Res. 2009;37:D175-80. https://doi.org/10.1093/nar/gkn815
42. Jacobs S, Schürmann A, Becker W, Böckers TM, Copeland NG, Jenkins NA, *et al*. The mouse ADP-ribosylation factor-like 4 gene: two separate promoters direct specific transcription in tissues and testicular germ cell. Biochem J. 1998;335:259-65. https://doi.org/10.1042/bj3350259
43. Finley JW. Reduction of cancer risk by consumption of selenium-enriched plants: enrichment of broccoli with selenium increases the anticarcinogenic properties of broccoli. J Med Food. 2003;6:19-26. https://doi.org/10.1089/109662003765184714

44. Finley JW, Sigrid-Keck A, Robbins RJ, Hintze KJ. Selenium enrichment of broccoli: interactions between selenium and secondary plant compounds. J Nutr. 2005;135:1236-8. https://doi.org/10.1093/jn/135.5.1236

45. Qazi IH, Angel C, Yang H, Zoidis E, Pan B, Wu Z, *et al*. Role of selenium and selenoproteins in male reproductive function: a review of past and present evidences. Antioxidants. 2019;8:268. https://doi.org/10.3390/antiox8080268

46. Kheradmand N, Kamkar R, Moshajjari M, Baazm M. Effect of selenium and pentoxifylline on expression of CATSPER1 and 2 genes and FSH/LH levels in treated mice by dexamethasone. Andrologia. 2019;51:e13279. https://doi.org/10.1111/and.13279

47. Wang HF, Chang M, Peng TT, Yang Y, Li N, Luo T, *et al*. Exposure to cadmium impairs sperm functions by reducing CatSper in mice. Cell Physiol Biochem. 2017;42:44-54. https://doi.org/10.1159/000477113

48. Mohammadi S, Gholamin M, Mansouri A, Mahmoodian RS, Babazadeh B, Kebriaei SM, *et al*. Effect of cadmium and nickel on expression of CatSper 1 and 2 genes in mice. Toxin Rev. 2018;37:216-22. https://doi.org/10.1080/15569543.2017.1350192

49. Jiménez-Badillo SE, Oviedo N, Hernández-Guzmán C, González-Mariscal L, Hernández-Sánchez J. Catsper1 promoter is bidirectional and regulates the expression of a novel lncRNA. Sci Rep. 2017;7:1-2. https://doi.org/10.1038/s41598-017-13867-2

50. Mata-Rocha M, Hernández-Sánchez J, Guarneros G, de la Chesnaye E, Sánchez-Tusié AA, Treviño CL, *et al*. The transcription factors Sox5 and Sox9 regulate

Catsper1 gene expression. FEBS Lett. 2014;588:3352-60. https://doi.org/10.1016/j.febslet.214.07.024

51. Liu X, Zheng J, Xue Y, Qu C, Chen J, Wang Z, *et al*. Inhibition of TDP43-mediated SNHG12-miR-195-SOX5 feedback loop impeded malignant biological behaviors of glioma cells. Mol Ther Nucleic Acids. 2018;10:142-58. https://doi.org/10.1016/j.omtn.2017.12.001
52. Koh E, Wimalasiri KM, Chassy AW, Mitchell AE. Content of ascorbic acid, quercetin, kaempferol and total phenolics in commercial broccoli. J Food Compost Anal. 2009;22:637-43. https://doi.org/10.1016/j.jfca.2009.01.019
53. Jia Y, Lin J, Mi Y, Zhang C. Quercetin attenuates cadmium-induced oxidative damage and apoptosis in granulosa cells from chicken ovarian follicles. Reprod Toxicol. 2011;31:477-85. https://doi.org/10.1016/j.reprotox.2010.12.057
54. Moretti E, Mazzi L, Terzuoli G, Bonechi C, Iacoponi F, Martini S, *et al*. Effect of quercetin, rutin, naringenin and epicatechin on lipid peroxidation induced in human sperm. Reprod Toxicol. 2012;34:651-7. https://doi.org/10.1016/j.reprotox.2012.10.002
55. Ranawat P, Pathak CM, Khanduja KL. A new perspective on the quercetin paradox in male reproductive dysfunction. Phytother Res. 2013;27:802-10. https://doi.org/10.1002/ptr.4799
56. Liu CM, Ma JQ, Xie WR, Liu SS, Feng ZJ, Zheng GH, *et al*. Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-κB pathway. Food Chem Toxicol. 2015;82:19-26. https://doi.org/10.1016/j.fct.2015.05.001

57. Qiu W, Lin J, Zhu Y, Zhang J, Zeng L, Su M, *et al*. Kaempferol modulates DNA methylation and downregulates DNMT3B in bladder cancer. Cell Physiol Biochem. 2017;41:1325-35. https://doi.org/10.1159/000464435

58. Imran M, Rauf A, Shah ZA, Saeed F, Imran A, Arshad MU, *et al*. Chemo-preventive and therapeutic effect of the dietary flavonoid kaempferol: A comprehensive review. Phytother Res. 2019;33:263-75. https://doi.org/10.1002/ptr.6227

59. Zhang C, Su ZY, Khor TO, Shu L, Kong AN. Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. Biochem Pharmacol. 2013;85:1398-404. https://doi.org/10.1016/j.bcp.2013.02.010

60. Yang SH, Long M, Yu LH, Li L, Li P, *et al*. Sulforaphane prevents testicular damage in Kunming mice exposed to cadmium via activation of Nrf2/ARE signaling pathways. Int J Mol Sci. 2016;17:1703. https://doi.org/10.3390/ijms17101703

61. Alkharashi NA, Periasamy VS, Athinarayanan J, Alshatwi AA. Assessment of sulforaphane-induced protective mechanisms against cadmium toxicity in human mesenchymal stem cells. Environ Sci Pollut Res. 2018;25:10080-9. https://doi.org/10.1007/s11356-018-1228-7

62. Liang J, Hänsch GM, Hübner K, Samstag Y. Sulforaphane as anticancer agent: A double-edged sword? Tricky balance between effects on tumor cells and immune cells. Adv Biol Regul. 2019;71:79-87. https://doi.org/10.1016/j.jbior.2018.11.006

Table 1 The sequence of the designed primers used for RT-PCR

Gene	Function	Accession number	Primer sequences	Product size	Optimized cycle number*	Reference
CatSper1	Sperm motility	NM_139301.3	Forward: 5'-'TCGGAGAACCACAGAGAAGAG-3'	566 bp	36	(27)
			Reverse: 5'-CACACACCGGGAATATCTTC-3'			
CatSper2	Sperm motility	NM_153075.3	Forward: 5'-TGGCCACAGAGCAGTATTTG-3'	513 bp	36	(27)
			Reverse: 5'-TGTCAGGCTGTTGCTTTGTC-3'			
Arl4a	Spermatogenesis	NM_007487.3	Forward: 5'-CAGGCTGCAGTTCAACGAAT-3'	377 bp	30	Current study
			Reverse: 5'-AATGCCAAGGAGTCGATGAG-3'			
Sox5	Transcription factor	NM_001113559.2	Forward: 5'-CCCCACATAAAGCGTCCAATG-3'	196 bp	32	(28)
			Reverse: 5'-TCTCCAGGTGCTGTTTGCTGAG-3'			
Sox9	Transcription factor	NM_011448.4	Forward: 5'-GAAGCTGGCAGACCAGTACC-3'	479 bp	36	(29)
			Reverse: 5'-CTGCTCAGTTCACCGATGTC-3'			
β actin	Reference gene	NM_007393.1	Forward: 5'-'GGGAAATCGTGCGTGACAT – 3'	385 bp	30	(27)
			Reverse: 5'-TCAGGAGGAGCAATGATCTTG -3'			

* The number of PCR cycles was determined to obtain a detectable signal without reaching saturation.

Table 2 Body and testis weight in NMRI male mice treated with 200 and 400 µl of broccoli extract (BE) of (1 g ml^{-1}) with and without Cd (1 mg kg^{-1} mouse weight). Data represent the mean of six mice in each group (± SD). Different letters indicate significant difference among groups ($p \leq 0.05$; Oneway ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

Sample	Control	Cd	BE (200 µ)	Cd+ BE (200 µ)	BE (400 µ)	Cd+ BE (400 µ)
Body weight (g)	47.7±3.9a	39.7±5.2a	38.7±4.8a	44.8±5.7a	40.9±1.5a	45.2±5a
Testis weight (g)	0.17±0.03a	0.16±0.04 a	0.13±0.01a	0.15±0.03a	0.14±0.02 a	0.16±0.02a

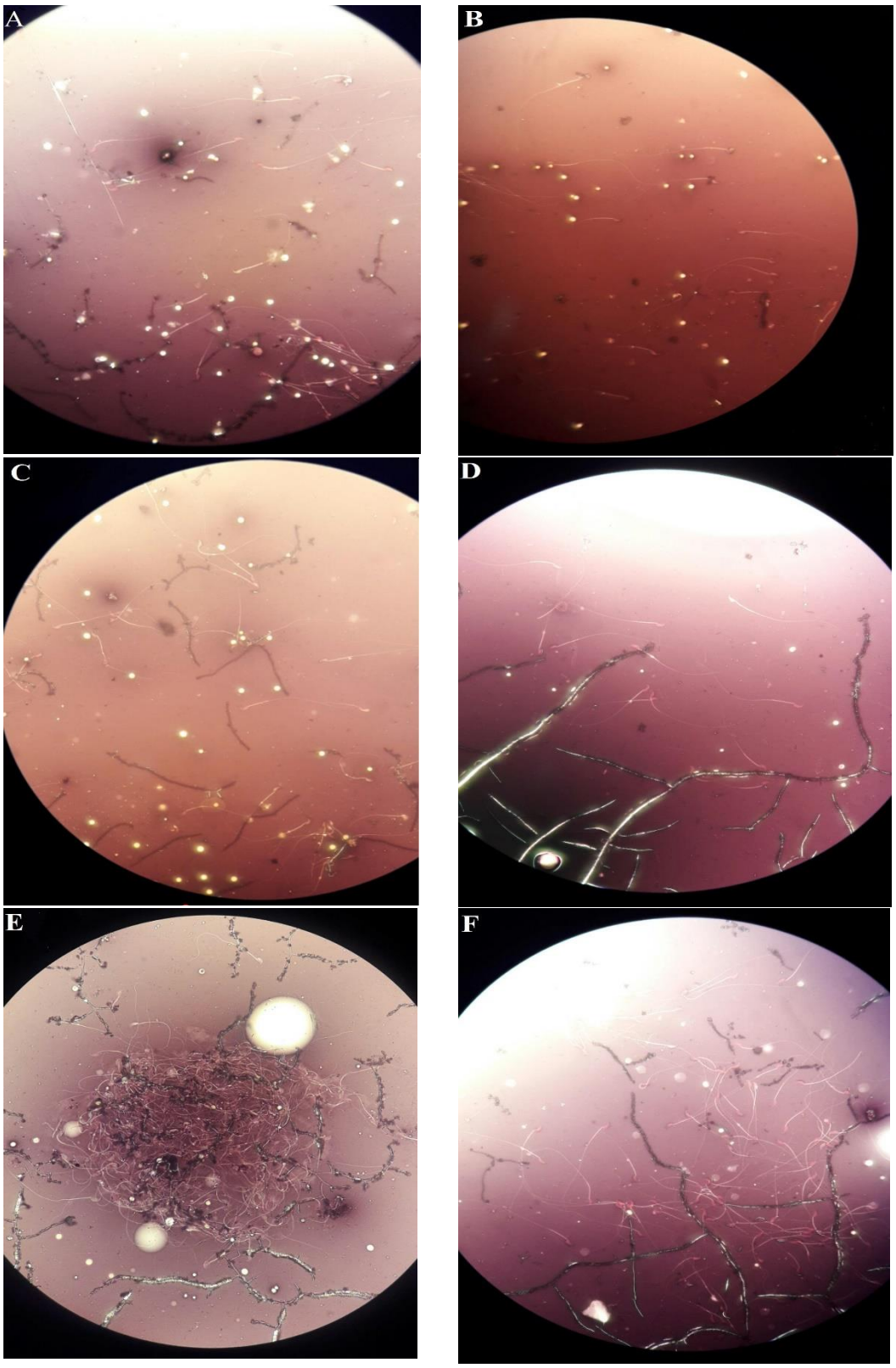

Figure 1. Microscopic images of sperm in NMRI mice treated with Cd and BE.

A: Control, B: Cd, C: 200 µl of BE (1 g ml^{-1}), D: 200 µl of BE (1 g ml^{-1}) plus Cd (1 mg kg^{-1}), E: 400 µl of BE (1 g ml^{-1}), F: 400 µl of BE (1 g ml^{-1}) plus Cd (1 mg kg^{-1}). With a magnification of 4×

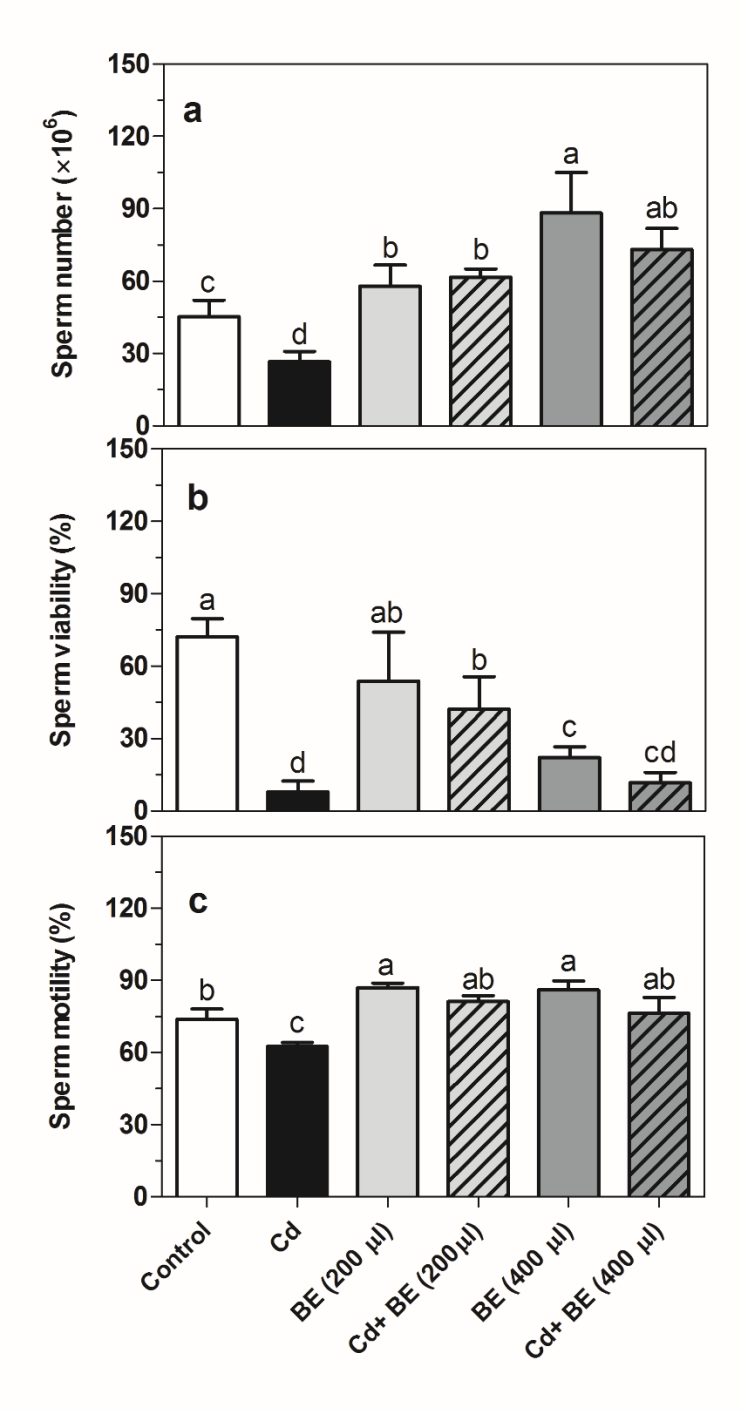

Figure 2. Sperm count (a), sperm viability (b) and sperm motility (c) in NMRI male mice treated with 200 and 400 µl of BE (1 g ml^{-1}) with and without Cd (1 mg kg^{-1} mouse weight). Data represent the mean of six mice in each group (± SD). Different letters indicate significant difference among groups ($p \leq 0.05$; One way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

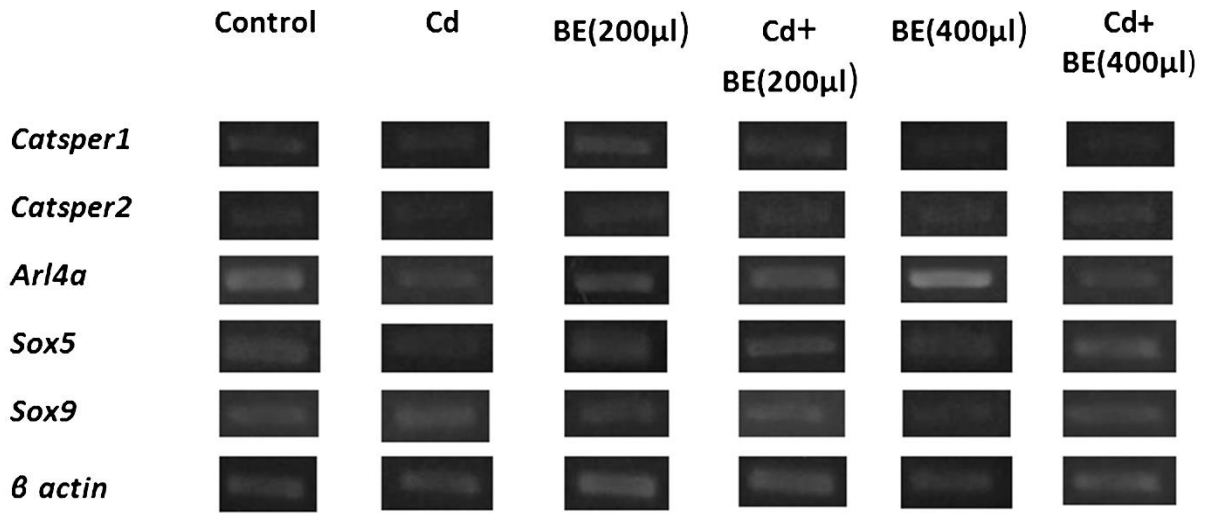

Figure 3. Relative gene expression determined by semi-quantitative RT-PCR. Expression of genes corresponds to the ratio of the target gene divided by the reference gene (β actin).

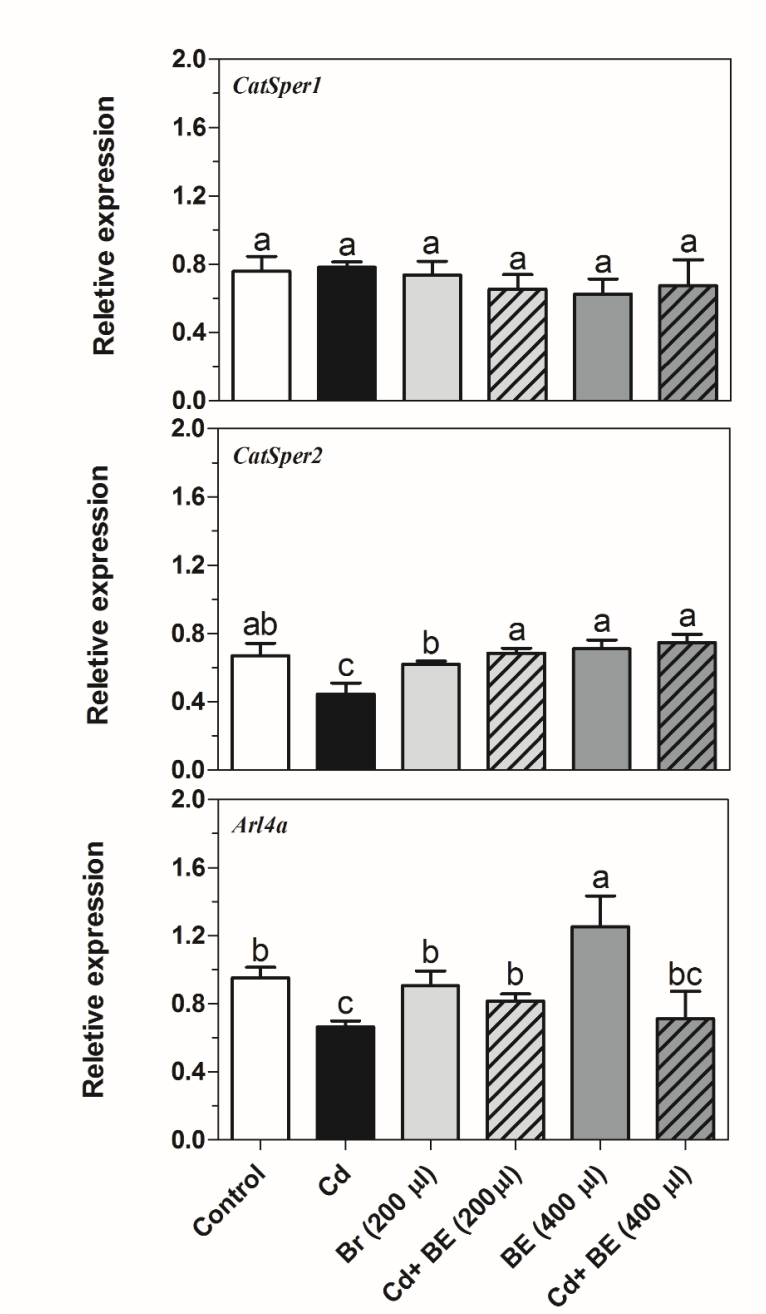

Figure 4. Transcript levels of *Catsper1* and *Catsper2* (genes involved in sperm motility) as well as *Arl4a* (gene involved in sperm count) in NMRI male mice. For experimental details, see legend of Figure 1. Relative gene expression was determined by RT-PCR compared to β actin as a reference gene. Data represent the mean of three mice in each group (± SD). Different letters indicate significant difference among groups ($p \leq 0.05$; One way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

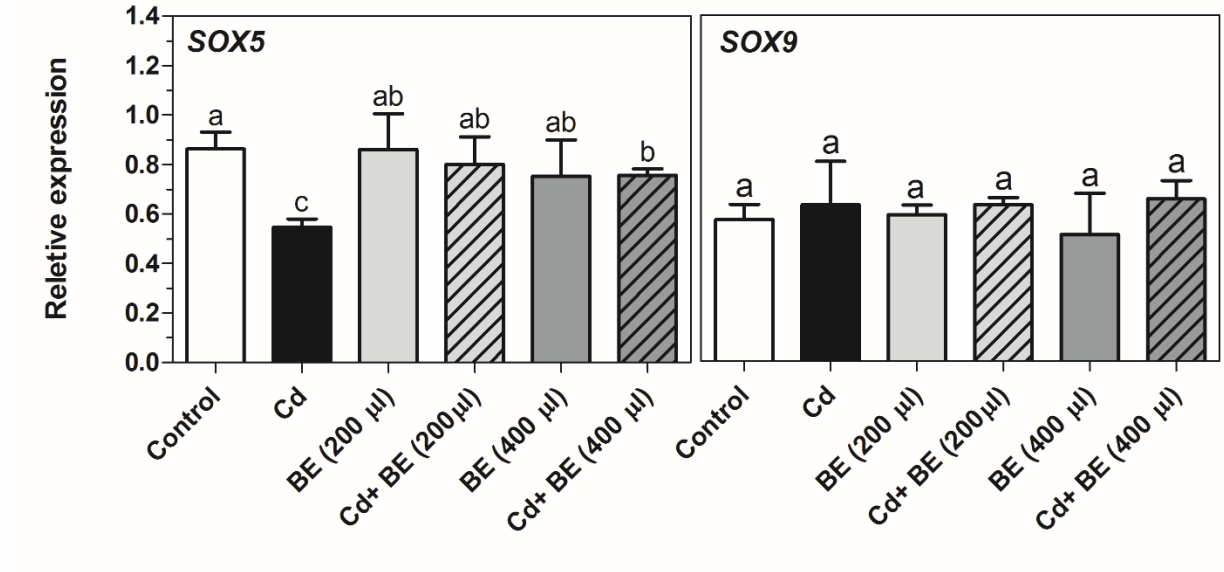

Figure 5. Transcript levels of *Sox5* and *Sox9* (transcription factors involved in sperm motility) in NMRI male mice. For experimental details, see legend of Figure 1. Relative gene expression was determined by RT-PCR compared to β actin as a reference gene. Data represent the mean of three mice in each group (± SD). Different letters indicate significant difference among groups ($p \leq 0.05$; One way ANOVA; Tukey´s HSD all-pairwise comparisons as a post-hoc test).

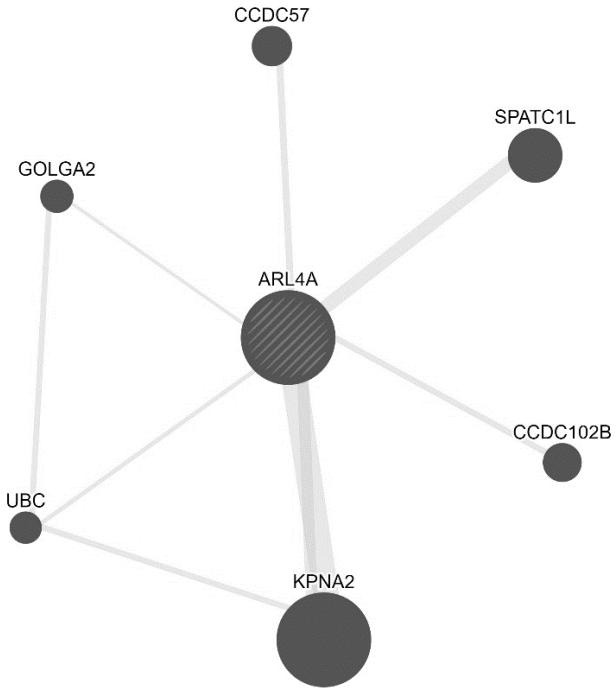

Figure 6. Protein-Protein interaction between human ARL4A protein and six other proteins based on GeneMANIA algorithm.