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Original Article

Effect of Long-Term Exposure to Shallow Well Water on Semen Parameters and mRNA Levels of Spermatozoa Tail Formation-Responsible Genes in Rams

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Abstract

Introduction: Exposure to heavy metals is linked to impaired animal fertility through disruptions in gene expression in spermatozoa. However, the mechanisms behind these effects are not fully understood. This study aimed to assess the impact of long-term consumption of shallow well water contaminated with heavy metals on semen quality and mRNA expression of genes related to sperm tail formation in rams.

Materials and Methods: Eighty sexually mature rams were enrolled, including 40 from four locations in Al-Diwaniyah governorate (Al-Shamiya, Al-Dughara, Al-Hamza, and Al-Shanafiya), which had been drinking shallow well water since birth (experimental group), and 40 drinking municipal tap water (control group). Water samples were analyzed for pH, total dissolved solids, salinity, turbidity, and heavy metal content (cadmium, lead, mercury). Semen was collected using an artificial vagina and evaluated by computer-assisted sperm analysis (CASA). Total RNA was extracted from spermatozoa, and the expression of *CATSPER*, *AKAP4*, and *SPAG6* genes was quantified using one-step real-time PCR with SYBR Green.

Results: Shallow well water had significantly higher turbidity, salinity, dissolved solids, and heavy metal concentrations compared to tap water ($p \leq 0.05$). Rams drinking well water showed a significantly lower percentage of motile sperm ($p < 0.001$). Additionally, the expression levels of *CATSPER*, *AKAP4*, and *SPAG6* genes were significantly reduced in the well water group compared to the control group ($p < 0.001$). Specifically, *CATSPER* expression decreased by 85.5%, *AKAP4* by 82.8%, and *SPAG6* by 87.4% in the well water group relative to controls.

Conclusions: Chronic exposure to contaminated shallow well water markedly impairs sperm motility and downregulates key genes involved in tail formation in rams, emphasizing the reproductive risks of heavy metal pollution.

Keywords: Shallow Well Water, Semen Quality, Gene Expression, Ram Fertility

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Introduction

Climate change and water shortages are reducing the availability of suitable water for farm animals. In Iraq, many farmers rely on well water to irrigate their animals due to the low water levels of the Tigris and Euphrates rivers and the lack of rainfall in recent decades.[1] One of the affected areas in central Iraq is the Al-Diwaniyah Governorate, leading to increased dependence on shallow wells that collect water from the surface, making them vulnerable to pollution from environmental contaminants such as septic tanks common in rural areas, or indirect sources like fertilizers and pesticides used in agricultural areas or polluted rainwater.[2] The irrigation of animals in these areas primarily relies on well water, making it a crucial factor for the productivity and health of the animals.[3] The important heavy metals present in drinking water, along with their guideline values set by WHO,[4] include cadmium (Cd), lead (Pb), and mercury (Hg), which are spread due to industrial pollution and soil erosion. Watering animals for long periods may cause an increase in the accumulation of some of them, which causes harmful effects on the animal's health and decreased fertility.[5] This has been supported by many previous studies that confirmed the effect of polluted well water on hormonal balance and sperm quality in males. This effect was also confirmed by Manouchehri et al,[6] who that showed that exposure of male mice to water contaminated with cadmium and lead led to a decrease in testosterone concentration, sperm count and motility. The main reason for these changes may be the difference in gene expression and enzymatic activity in the epididymis, which causes a decrease in the ability of the testicle to produce steroids, resulting in a decrease in sperm vitality.[7]

The interactions between different heavy metals and transcription factors linked to metal toxicity may contribute to the variations in gene expression observed in living cells

after exposure to different heavy metals.[8] Oxidative stress, mostly caused by heavy metals, is one of the main causes of impaired sperm motility. Reactive oxygen species (ROS) generated by heavy metals damage sperm DNA and sperm cell membranes.[9] They can impact sperm motility in sheep, similar to how they affect sperm from humans and other animals.[10] Cadmium induces oxidative stress and lipid peroxidation in sperm cells. It can disrupt the endocrine system, leading to decreased testosterone levels, reduced sperm motility, increased DNA damage, and lowered sperm viability. Lead poisoning interferes with the antioxidant defense system, causing oxidative stress. It disrupts calcium signaling and damages the sperm cell membrane, resulting in reduced sperm motility, abnormal sperm morphology, and decreased sperm concentration. Moreover, mercury contamination of well water leads to oxidative stress and damage to the mitochondria of sperm cells, causing ATP deficiency, decreased sperm motility and vitality, and an increase in the percentage of deformed sperm.[11]

One of the most important genes that control sperm motility is the cation channel-associated sperm (*CATSPER*4) gene, which is located in the sperm tail membrane.[12] These channels are vital for regulating the flow of calcium ions into the tail, which is essential for sperm hyperactivation. This hyperactivation is essential for egg penetration and successful fertilization. Therefore, any defect in this gene directly affects the sperm's ability to swim effectively.[13] The A-kinase Anchoring Protein 4 *(A-KINASE 4*) gene plays a major role in maintaining the integrity and function of sperm by aiding in sperm movement and increasing production capacity.[14] It acts as a scaffold or anchoring hub for the assembly of numerous enzymes and regulatory proteins, including those involved in energy metabolism. Therefore, it not only supports the structural integrity of the tail, but also regulates the energy production needed for motility and sperm function.[15] The Sperm-Associated Antigen 6 (*SPAG6*) gene is considered one of the important genes encoding a special protein that forms the basic structure of the sperm tail, regulating sperm movement.[16] SPAG6 is an essential protein for the formation and stability of the axoneme, the motile core of the sperm tail composed of microtubules. It plays a critical role in regulating the assembly of these microtubules and maintaining their cohesive structure, essential for coordinated and efficient tail motility. Any defect in SPAG6 can lead to structural defects in the tail, resulting in impaired or completely absent motility.[17]

Some heavy metals can act as "endocrine disruptors," interfering with the action of hormone receptors (such as androgen or estrogen receptors) that regulate the gene expression of many proteins necessary for sperm formation.[18] Heavy metals can bind directly to proteins, including transcription factors and enzymes involved in transcription and translation. This binding can alter their shape or function, leading to inhibition or alteration of gene expression regulation of target genes.[19] Oxidative stress can lead to DNA damage and chemical modifications to nucleotides that can affect DNA stability and transcriptional regulation. It can also affect the activity of transcription factors, which bind to DNA to regulate gene expression.[20] Metals such as cadmium and lead can mimic or interfere with calcium ions, affecting calcium-dependent signaling pathways that play a role in regulating gene expression and sperm motility such as the *CATSPER* gene.[21]

The main objective of this study is to clarify the effect of long-term exposure to heavy metals that contaminate shallow well water used for watering animals on sperm characteristics and motility. In addition to focusing on measuring the expression of some important genes that participate in the formation and development of sperm tails in rams, three genes were identified by searching the GenBank website by typing the keywords (genes responsible for sperm tail formation). These genes were chosen for their importance in most studies (*CATSPER*, *AKAP4,* and *SPAG6*). We hypothesize that chronic exposure to heavy metals in shallow well water reduces sperm motility through the downregulation of sperm tail-related genes.

Materials and Methods

Animals and Study Area

All procedures involving the animals were carried out without any surgical intervention and without attempting to kill the animal. This study complied with the Guidelines for the Ethical Use of Animals and the Ethical Committee of the Faculty of Biotechnology, University of Al-Qadisiyah, Iraq. The experiment period was from February to July 2024. The study was conducted in Al-Diwaniyah governorate, located in the southern part of Iraq (middle of the Euphrates region), geographically bound by latitude Exact geographical coordinates: 31º 56′ 28.8″ N, 044º 54′ 12.6″ E. Four severely affected areas (Al-Shamiya, Al-Dughara, Al-Hamza, and Al-Shanafiya) were identified based on several reports from sheep farmers of clinical signs indicative of heavy metal poisoning, such as a decrease in ram fertility, increased abortion rates, and premature births, in addition to general symptoms such as weakness, lethargy, weight loss, decreased appetite, and increased thirst. After a comprehensive investigation, we found that these animals had been drinking shallow well water since birth (Figure 1).

Study Design

In the study areas, we randomly selected forty sexually mature rams of the local Awassi breed, all of which had been drinking shallow well water since birth. These animals were matched in terms of age (18-22 months) and weight (30.5 to 37.25 kg) and were considered as the experimental group. Additionally, 40 rams were selected from the same

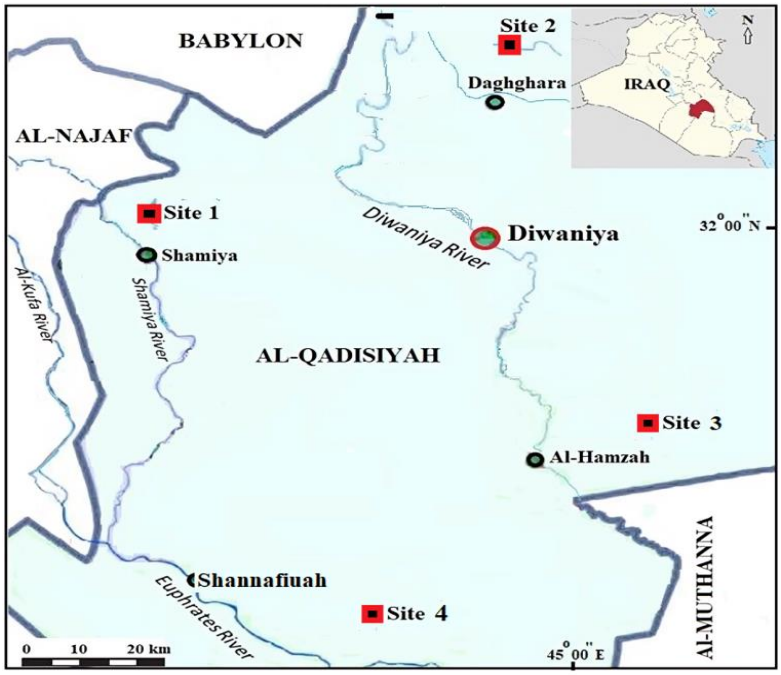

Figure 1. Map of Al-Diwaniyah Governorate Showing the Four Well Sites (GPS): Site 1: Al-Shamiya, Site 2: Al-Dughara, Site 3: Al-Hamza, Site 4: Al-Shanafiya.

areas that were drinking tap water, forming the control group. The ages of the control group ranged from 17 to 24 months, and their weight was between 35.5 and 40.5 kg. The sample size of 40 rams per group was chosen based on the availability of suitable animals and consistency with similar animal studies in the literature, aiming to ensure sufficient statistical power for detecting significant differences between groups.

Water Sampling and Analysis

During the summer season (May-July), water samples were collected from experimental areas using polyethylene bottles as described by Abbas and Hussein.[22] We measured pH, total dissolved solids (TDS) in mg/L, and salinity using Pocket Testers (PCTSTestr™ 5 Pocket Tester, AKTON®, USA) according to the manufacturer's recommendations. Additionally, turbidity was measured in Nephelometric Turbidity Units (NTU) using a turbidity meter (Turbidity Meter, Benchtop Type, BEP-TB200, INFITEK®, China) in accordance with the manufacturer's instructions. The levels of heavy metals (cadmium, lead, and mercury) in water samples were analyzed according to Mohan et al.[23] using a portable water quality analyzer based on the standard method called anodic stripping voltammetry (ASV) (SKYRAY Instrument, Portable Heavy Metal Analyzer, HM300P, China).

CASA Technique

We collect semen using artificial vagina (AV) with estrous sheep as teasers, following the method described by Santolaria et al.[24] We evaluate semen parameters and morphological index using Computer-Assisted Sperm Analysis (CASA) with the CEROS II® device from Animal Semen Analysis, Zeiss, IMV-Technologies Co. France. The sperm chamber used is Leja products BV® 4 Chamber Slides with a depth of 20 µm from IMV-Technologies Co. France. The semen parameters measured include ejaculation volume, pH, concentration ($\times 10^9$/ml), total sperm count ($\times 10^9$/ejaculation), total sperm motility, progressive motility, normal sperm morphology index, and sperm vitality.

The spermatozoa morphology index was assessed by calculating the percentage of normal head, neck, and tail index using the staining kit (SpermFunc® Diff-Quik-Staining Kit for Spermatozoa Morphology, chain). The sample volume was varied (between 5 µl) based on the concentration for the smear preparation, yielding 4 to 10 spermatozoa per field when seen through a 100x oil immersion microscope during morphometric analysis. As previously mentioned, the air-dried smears were fixed in a fixative solution and stained with Diff Quick dye. With bright field optics and a ×100 oil immersion objective, at least 100 stained spermatozoa were examined.[25]

Isolated of Spermatozoa

We isolated the sperm cells from seminal plasma according to Schellhammer et al.[26,27] Briefly, the seminal plasma was washed with 1 ml of (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) (HEPES) Buffer (Capricorn Scientific GmbH, Germany), then eliminated the somatic cells by Somatic Cell Lysis buffer (SCLB) and stored on ice for 30

min. A purified spermatozoa pellet was obtained by light microscopy and stored at -80 ℃ until RNA extraction.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from spermatozoa cells using a total RNA isolation kit (Thermo Scientific® GeneJET RNA Purification Kit, California, USA), and DNA removal was achieved by adding DNase I (Thermo Scientific® RNase-free kit, California, USA). The concentration and purity of these RNAs were determined by measuring the A260/280 nm ratio using a nano-drop spectrophotometer (OPTIMA®, SP-3000 Nano, UV/V spectrophotometer, Tokyo, JAPAN). Gel electrophoresis was performed on an agarose gel to assess the integrity of the RNA, with the presence of two distinct bands indicating intact and undegraded RNA. The absence of "smearing" on the gel indicates good quality, as described by Slater.[28] The RNA samples were adjusted to the same concentrations using diethylpyrocarbonate (DEPC) water for each sample.[29]

Complementary DNA (cDNA) was synthesized from the RNA samples using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with random primers, following the manufacturer's instructions of the Thermo Scientific® RevertAid First Strand cDNA synthesis Kit, California, USA. Specific primer sequences were designed using the GenBank database from the National Center for Biotechnology Information (NCBI), and PCR Primer Stats (http://www.bioinformatics.org/sms2/pcr_primer_stats.html) was used to check each designed primer pair for the possibility of dimer formation (Table 1). The mRNA of target genes (*CATSPER*, *AKAP4*, and *SPAG6*) expression was quantified by one-step real-time PCR using thermal reaction by (Excecycler 96® Thermal cycler for real-time PCR, Bionner, Korea), with SYBR Green Master Mix (AccuPower® Greenstar™ qPCR PreMix, Bionner, Korea). The stably expressed housekeeping gene was glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).[30] The qPCR assay was performed in a total reaction mixture of 25 µl containing (5 µl of RNA (equivalent to 100 ng of total RNA), 12.5 µl of 2× Quantities multiplex RT-PCR master mix, 0.25 µl of RT-mix, 200 nM each forward and reverse primers. The thermal profile included an initial denaturation and polymerase activation step for 15 min at 95 °C, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each qPCR assay contained template-negative and reverse transcriptase-negative samples as controls, and all samples were run in duplicate by formula $2^{-\Delta CT}$ of the target gene.[31]

Table 1. The Primers Sequence, Fragment Size (bp) and Melting Temperatures (Tm) Used for the Target Genes Including *CATSPER, AKAP4,* and *SPAG6*

Gene Name	Gene ID	Primer sequences	Amplicon size (bp)
CATSPER	101107374	F: 5′ GGGTTGCCATTTGCTTCTTC 3′ R: 5′ CACACACACACACACACATAAC 3	120
AKAP4	101102535	F: 5′ CTGAAGAATCCCAAGGACAGAG′ R: 5′ GTGAAGAGGTAGTGAGCGTTTAG 3′	101
SPAG6	101103745	F: 5′ GTCCTCCATAGTGGCTGTACTA 3′ R: 5′ CCGCCATCTCAAAGTCTACAA 3′	109
GAPDH	443005	F: 5′ GAGTAAGTGTGGGAGATGGAAC 3′ R: 5′ GCCTATGAGAAAGACAGGACAA 3′	119

F: Forward primer; R: Revers primer

Statistical Analysis

All data are presented as means ± Standard Error of the Mean (SEM). To assess statistical differences between the tap water group and the shallow well water group for each water analysis parameter, ANOVA was performed, and the Tukey's Honest Significant Difference (HSD) post-hoc test was used for multiple comparisons when ANOVA indicated a significant overall effect. Differences were considered statistically significant at a p-value less than 0.05. The gene expression results were expressed as means ± standard error of the mean (Mean ± SEM). To assess differences in gene expression between the control and experimental groups, an independent two-sample t-test was used. Assumptions of normal distribution and equality of variances were verified. Differences were considered statistically significant when $p < 0.05$. The statistical package of STATISTICA 6.0 (Statsoft Inc., Tulsa, OK, USA) was used for the foregoing analyses.[32]

Results

Physical Water Parameters

Table 2 estimates the association between tap water and shallow well water. The pH of tap water is neutral (7.21 ± 0.07), while shallow well water is highly acidic (5.508 ± 0.48). This significant difference (p = 0.038) indicates potential environmental or contamination issues affecting the well water. Tap water had low turbidity (6.103 ± 0.603), indicating it was relatively clear. In contrast, shallow well water had extremely high turbidity (53.066 ± 6.349), suggesting it was highly significant (p = 0.005) and likely contained many suspended particles. The salinity (ppt) of tap water was 0.403 ± 0.09, and in shallow wells was 13.628 ± 1.727. The shallow well water salinity was higher than that of tap water (p = 0.005), indicating that the well water is significantly more saline, which could affect its suitability for animal drinking. The TDS mg/L of tap water was 660.14

± 45.122, yet in the shallow wells were 5692.75 ± 665.25. The shallow well water TDS was higher (p = 0.004), indicating that the well water contains more dissolved substances, which could affect its taste, quality, and safety.

Table 2. Comparison of Physicochemical Parameters and Heavy Metal Concentrations in Tap Water and Shallow Well Water

Water analysis	Tap Water (n = 40) (mean ± SEM)	Shallow well (n = 40) (mean ± SEM)	*p*-values	Standard Value (33)
pH	7.2 ± 0.07	5.508 ± 0.48	0.038	6.5-8.5
Turbidity (NTU)	6.103± 0.603	53.066 ± 6.349	0.005	≤ 6 NTU
Salinity (ppt)	0.403 ± 0.09	13.628 ± 1.727	0.005	
Total dissolved solids (TDS) mg/L	660.14 ± 45.122	5692.75± 665.25	0.004	400
Cadmium (Cd) mg/L	0.017 ± 0.0013	1.525 ± 0.127	0.001	0.003
Lead (Pb) mg/L	0.0134 ± 0.0049	1.2623 ± 0.097	0.001	0.01
Mercury (Hg) mg/L	0.0028 ± 0.00048	0.079 ± 0.0058	0.001	0.001

NTU: Stands for nephelometric turbidity unit; ppt: Parts per thousand; SEM: Standard error of the mean; p ≤ 0.05 was considered of statistical significance.

Heavy Metals Analysis

Cadmium, lead, and mercury concentrations in tap water were 0.017 ± 0.0013, 0.0134 ± 0.005, and 0.0028 ± 0.00048 mg/L, respectively. However, in the shallow well, they were 1.525 ± 0.127, 1.2623 ± 0.097, and 0.079 ± 0.0058 mg/L, respectively. The concentration of these heavy metals in the shallow well water is significantly higher (P = 0.001), indicating potential harm from cadmium contamination that exceeds safe drinking water standards. Mercury levels in shallow well water are also elevated compared to tap water, and even small amounts of mercury can be toxic. Additionally, the lead concentration is highly toxic, especially at this level, which far exceeds safe limits, making this well water unsafe for consumption.

Semen Analysis

The semen analysis results in Table 3 compare various parameters between samples from control rams and experimental rams exposed to shallow well water. In control rams, the mean semen pH value was 7.10 ± 0.058, while in experimental animals, it was 6.46 ± 0.033. These pH values were significantly lower in the animals exposed to shallow

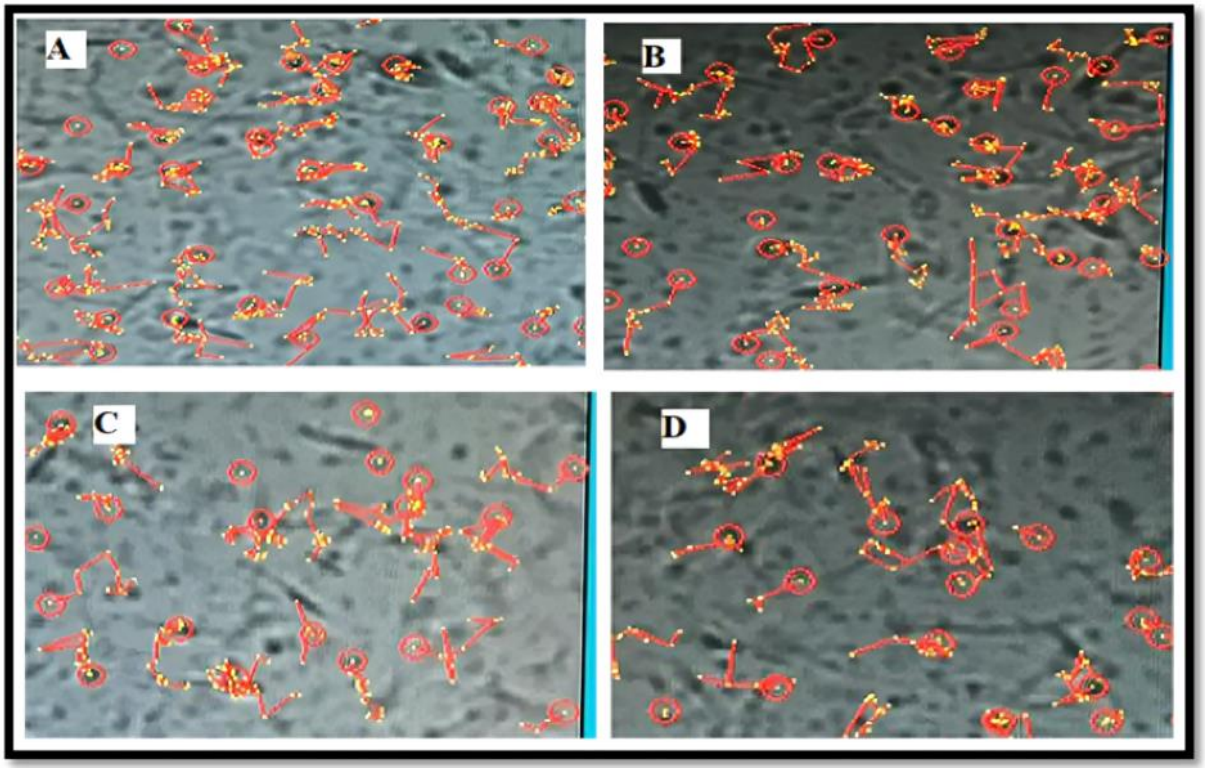

Figure 2. Assessment of Sperm Motility by CASA System: Comparative Analysis of Rapid Progressive Sperm Tracks. (A) Control group sample shows a relatively high density of progressive, fast-moving sperm tracks characterized by longer, more linear red spiral tracks, indicating continuous and efficient forward motility. (B) shows a high and constant density of sperm tracks. (C) Experimental group sample: The red spiral tracks appear shorter and less linear, indicating reduced progressive motility and possibly more erratic or less efficient motility patterns. (D) shows a significantly lower density of progressive, fast-moving sperm tracks and more instances of discontinuous or fragmented red spiral tracks, indicating impaired progressive motility and altered motility dynamics within the experimental group.

Table 3. Comparison of CASA-Analyzed Sperm Motility Parameters between Control and Experimental Groups

Semen Analysis	Control group (n = 40) (mean ± SEM)	Experimental group (n =4 0) (mean ± SEM)	*p*- values
pH	7.10 ± 0.058	6.46 ± 0.033	0.001
Volume	1.27 ± 0.0359	1.00 ± 0.059	0.001
Concentration 10^9/ml	3.15 ± 0.247	2.22 ± 0.165	0.001
Morphology index % normal sperms	77.12 ± 3.00	45.17 ± 2.713	0.001
Total number of sperm count 10^9/ejaculation	3.99 ± 0.309	2.19 ± 0.186	0.001
Total sperm motility %	82.33 ± 1.731	52.27 ± 2.892	0.001
Progressive motility %	74.73 ± 2.275	25.67 ± 1.021	0.001

SEM: Standard error of the mean; $p \leq 0.05$ was considered statistically significant.

well water, indicating increased acidity, and this difference is highly statistically significant at ($p < 0.001$). In control rams, the semen volume was 1.27 ± 0.0359 ml, the sperm concentration (10^9/ml) was 3.15 ± 0.247, and the normal morphology index was 77.12% ± 3.00. In the experimental group, these values were 1.00 ± 0.059 ml for semen volume, 2.22 ± 0.165 for sperm concentration, and 45.17% ± 2.713 for the normal morphology index. The semen volume is significantly lower in the shallow well group than in the normal group, with a low *p*-value (0.001) indicating a statistically significant difference. The sperm concentration and morphological index are significantly lower in the shallow well group at ($p \leq 0.01$), indicating a statistically significant difference. According to CASA results, the total number of sperm (10^9/ejaculation) in normal samples was 3.99 ± 0.309. Yet, in the experimental samples, it was 2.19 ± 0.186. Total sperm motility in normal and experiment samples were 82.33 ± 1.731 and 52.27 ± 2.892, respectively, and the progressive motility values was 74.73 ± 2.275 and 25.67 ± 1.021, respectively. The percentage of progressively motile sperm was significantly lower in the shallow well group, with a highly significant difference at ($p \leq 0.001$) (Figure 2).

Gene Expression

The gene expression analysis was performed to compare the expression levels of target genes between the control and experimental groups using the 2ΔCt method. The gene expression values of the *CATSPER*, *AKAP4*, and *SPAG6* genes in the control group were 3.82 ± 0.186, 2.242 ± 0.216, and 2.525 ± 0.286 respectively, while in the experimental group, they were 0.553 ± 0.0712, 0.386 ± 0.055, and 0.319 ± 0.028 respectively. The expression levels of these target genes were significantly reduced in the experimental group exposed to shallow well water compared to the control group, with high significance at ($p \leq 0.001$). These findings indicate that exposure to shallow well water has a significant impact on the expression of specific genes related to sperm function (Figure 3).

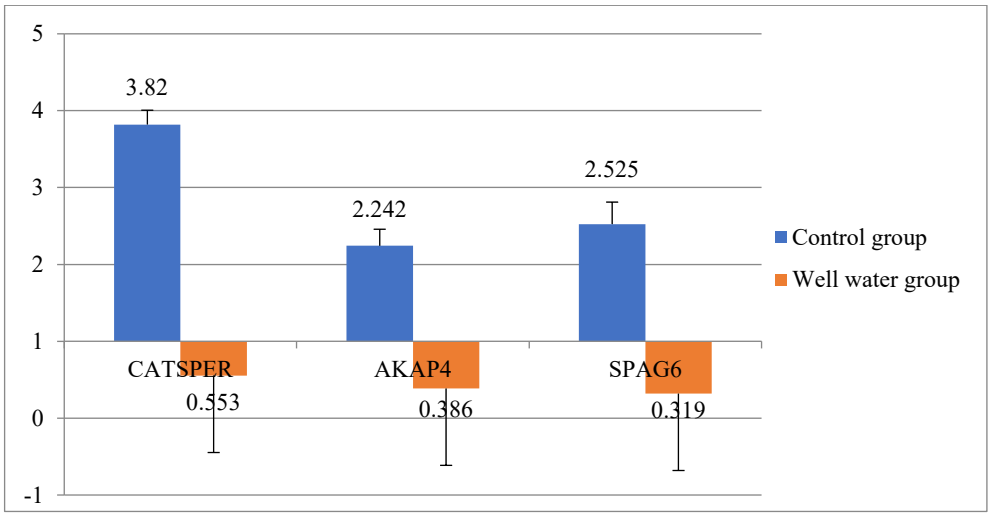

Figure 3. Relative Quantification of Target Gene Expression in Spermatozoa Cells of the Control and Well Water Groups. The bars shown represent the means of results from three independent experiments. The standard error of the mean is plotted. The 2-ΔCt method was used to calculate fold changes. The values represent median fold change with interquartile ranges; Asterisks indicate significant values ($p \leq 0.05$).

Discussion

The current study highlights the detrimental effects of long-term exposure to shallow well water contaminated with heavy metals on the reproductive health of rams. Our study did not isolate specific heavy metals but addressed the impact of this water on semen quality and sperm formation. This aims to understand the mechanism of action of these toxic substances and their effect on the RNA levels of

certain genes responsible for sperm tail formation, such as *CATSPER*, *AKAP4*, and *SPAG6*, to design new strategies to counteract the toxicity of heavy metals and their harmful effects on reproductive health in rams.

Table 2 shows some physical and chemical properties of shallow well water in Al-Dewaniya city. The pH levels in the well water samples were low ($p \leq 0.05$), indicating potential differences in chemical composition and quality compared to natural water. Many of these well water samples do not meet the World Health Organization standards for drinking water, as their acidic properties (pH less than 6.5) could potentially cause adverse environmental effects. These findings are consistent with the conclusions of Abanyie et al.,[34] which suggest that geological characteristics, pollution, and human activities likely impact groundwater sources, leading to acidosis that may result in weight loss, reduced production, decreased water and feed intake, digestive alterations, diarrhea, and poor feed conversion.

The analysis of shallow well water indicated a significant increase ($p \leq 0.05$) in the water turbidity level, indicating the presence of high concentrations of suspended particles and potential pollutants. This increase may be due to surface water runoff or human activities that have affected the groundwater in the study areas. The results of our study are consistent with Hussein et al.,[35] who confirmed the high level of turbidity in the water of some wells in Al-Diwaniyah governorate, Iraq. Additionally, the analysis of well water samples indicates high levels of salinity and total dissolved solids (TDS). According to WHO recommendations, safe drinking water should have a TDS value of no more than 400 mg/L, and the dissolved solids value should not exceed 1000 mg/L.[33] The well water pollution results from changes in natural geological processes, agricultural irrigation, industrial waste, and improper waste management.[36] The results of this study indicated high levels of salinity and TDS in shallow well water samples from all study areas. Previous studies have shown that these contaminants can directly affect health or indirectly reduce growth and production by decreasing overall water consumption.[37,38] The high rates of water evaporation may be due to high temperatures in the summer season, leading to an increase in the concentration of salts, which in turn causes an increase in the rate of dissolution of solids. In this study, the analysis of heavy metals (cadmium, lead, and mercury) in the target well water in Table 2 showed a significant difference ($p \leq 0.05$) compared to tap water samples. These results are consistent with many previous studies conducted by the American Public Health Association.[39] The acidic water will increase the concentration of heavy metals, suggesting an inverse relationship between cadmium toxicity and the pH of the aquatic environment, making these elements more soluble and thus more toxic.[40] The results of this study were in agreement with a previous study, which indicated that the warm well water in the same study area was highly saline and very hard, with a relatively high content of cadmium and lead in the targeted well water.[41]

According to CASA results in Table 3, the sperm parameters showed statistically low values ($p \leq 0.05$) in the rams exposed to shallow well water. The semen volume, pH, and sperm concentration showed significant differences compared with the control group, and these results agree with Kadirvel et al.[42] and Momeni and Eskandari,[43] which refer to the fact that high cadmium, mercury, and lead concentrations reduce the physiological function of many testicle cells (seminiferous tubules and Sertoli cells), and reduce all normal testes functions. The results in Figures 3 show a decrease in the expression of the target genes (*CATSPER*, *AKAP4*, and *SPAG6*) at a significance level of $p \leq 0.05$. This decrease indicates the effect of exposure to contaminated shallow well water on the molecular level of sperm cells. These results are consistent with several previous studies that demonstrate the effect of heavy metal pollution on the reproductive health of rams, such as Rzymski et al.[44] and Bhardwaj et al.[45]

The *CATSPER* channels play an important role in sperm formation; the sperm membrane undergoes modifications that activate calcium entry and increase its levels inside the cells, which helps improve sperm motility and function.[46] The results of our current study indicate that shallow well water contaminated with heavy metals affected the molecular level of sperm cells. Changing the expression of genes related to sperm structure and function led to decreased sperm motility and increased levels of morphological abnormalities. These results are consistent with the studies conducted by Villeneuve et al.[47] and Baseggio et al.,[48] which have suggested that prolonged exposure to heavy metals can alter the expression of specific genes in certain species, helping them adapt to environmental challenges.

The *AKAP4* gene is responsible for encoding a protein essential for sperm motility and tail integrity[49] by participating in the stabilization of protein kinase A (PKA) molecules, which is essential for controlling sperm motility.[50] The correct gene expression of *AKAP4* is important for the development of sperm motility, and any disturbance may lead to a decrease in sperm function and thus affect the fertility of the animals;[51] therefore, this gene has been considered a molecular marker for sperm quality in several species.[52] In our study, it was observed that high levels of lead and mercury in shallow well water samples could lead to decreased gene expression of *AKAP4* in experimental samples.

The *SPAG6* gene is important in infertility studies and treatment as it plays a fundamental role in sperm safety and helps in sperm motility development. Furthermore, any defect in its expression leads to sperm tail abnormalities that may cause infertility. The *SPAG6* gene expression occurs in

mature sperm cells.[53] In this study, the results of Figure 3 indicated a significant decrease ($p \leq 0.05$) in the gene expression of *SPAG6* in sperm cells in the group of rams exposed to well water compared to the control group. These results are consistent with the conclusions of previous studies.[54] Similar to cadmium poisoning, lead exposure can lead to decreased expression of the *SPAG6* gene, causing increased structural abnormalities in the sperm tail, resulting in poor sperm motility.[55] In previous studies like those by Wallace et al.[56] and Bellas et al.,[57] it was shown that heavy metals cause changes in the amounts of microRNA (miRNA), which controls the expression of many genes and signaling pathways, affecting the balance and function of mitochondria.

The current findings suggest that the decreased gene expression levels of *CATSPER*, *AKAP4*, and *SPAG6* may not only be due to the direct toxic effects of heavy metals on testicular cells but may also be linked to more complex molecular mechanisms. Oxidative stress resulting from the accumulation of metals such as cadmium, lead, and mercury can lead to the generation of reactive oxygen species (ROS), which damage proteins, lipids, and nucleic acids within sperm cells, ultimately inhibiting gene transcription and reducing the expression efficiency of genes regulating sperm motility.[58]

Methylation of THE promoter regions of these genes is also likely to contribute to their reduced transcriptional activity. Several studies have shown that long-term exposure to heavy metals causes epigenetic changes in reproductive tissues, leading to the silencing of some genes important for spermatogenesis.[59,60] In addition, there may be transcriptional interference or disruption of the binding of transcription factors to regulatory sites due to metal accumulation or its effect on the spatial structure of chromatin, inhibiting the normal transcription of target genes.[61] Accordingly, the observed changes in gene expression can be explained as a result of a complex interaction between oxidative stress, epigenetic changes, and transcriptional interference interconnected mechanisms that ultimately lead to poor sperm quality and decreased fertility. This disturbance can be explained by the high level of heavy metals in this water. Cadmium and lead poisoning are among the most important elements that many studies have been interested in investigating for their effect on hormone levels and reproductive failure.

However, this study has several limitations that should be noted. First, the study did not directly measure heavy metal concentrations in testicular tissue, which could have provided a clearer association between exposure levels and changes in gene expression. Second, the genetic analysis was limited to three key genes associated with sperm motility (*CATSPER*, *AKAP4*, and *SPAG6*), without addressing other genes that may be involved in oxidative stress processes or epigenetic regulation. Finally, the study relied on a limited sample size and a narrow geographic scope, which may limit the generalizability of the results. Therefore, future studies involving a larger number of genes, molecular pathway analyses, and histological examinations are recommended to more precisely elucidate the mechanisms underlying the toxic effects of heavy metals on reproductive functions in rams.

Conclusion

Water supply is considered the main source of heavy metal contamination. Thus, any physical or chemical changes in water affect the reproductive system physiology. Furthermore, heavy metals such as Cd, Pb, and Hg have adverse effects on spermatogenesis and sperm properties. The current study confirms that water contaminated with heavy metals diminishes normal sperm motility and increases sperm abnormality. Additionally, these negative outcomes have been linked to low specific tail gene expression (*CATSPER*, *AKAP4*, and *SPAG6*). Low expression may be due to an increase in reactive oxygen species (ROS) and oxidative stress, which can impair DNA repair proteins and cause tissue damage.

Authors' Contributions

AKA: Conceived and designed the study, performed data collection, and drafted the manuscript; OHH: Contributed to methodology, laboratory experiments, and data validation; QZB: Assisted in sample collection, molecular analysis, and interpretation of results. All authors read and approved the final manuscript.

Ethical Approval

The study protocol was reviewed and approved by the Animal Ethics Committee of the College of Biotechnology, University of Al-Qadisiyah, Iraq (Ethics Code: No. 1106. 2024.01.20).

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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