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Synthetic antioxidants mitigate oxidative sperm DNA fragmentation: Synthesis, characterization, and functional evaluation of Schiff's base complexes

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ABSTRACT

Oxidative stress (OS) is a primary contributor to sperm DNA fragmentation (SDF) and impaired male reproductive function. Numerous studies have demonstrated the advantages of natural antioxidants; however, evidence supporting the efficacy of synthetic antioxidants is limited and inconclusive. This study aimed to assess the effectiveness of laboratory-synthesized synthetic compounds in preserving sperm DNA and maintaining its integrity. We studied Schiff's base ligand [L] from L-asparagine and 4-dimethylaminobenzaldehyde, as well as its heavy metal complexes, utilizing FT-IR, UV–Vis, $^{1}H/^{13}C$-NMR, conductivity tests, and magnetic susceptibility analysis. We used the DPPH method to test how well the synthesized compounds worked as antioxidants. The Comet test was used to see how well the chemical protected sperm DNA. Hydrogen peroxide made sperm DNA fragmentation a lot worse. Treatment with the free Schiff base ligand [L] reduced damage in a dose-dependent way, with the 50 µg/mL dose bringing head DNA (HD%) back to roughly 92% ($P < 0.001$), which is the same as ascorbic acid. Metal complexation greatly increased protection. $[Mn_2(L).3Cl.H_2O]$ and $[Co_2(L).3Cl.H_2O]$ kept more than 99.9% of head DNA (HD%) and reduced tail DNA (TD%) to less than 0.12% ($P < 0.0001$ compared to the H_2O_2 group). The $Zn^{(II)}$ compound only protected about 80% of the head DNA (HD%). The DPPH IC_{50}values were in line with functional protection, which showed that Mn and Co complexes worked as antioxidants. Schiff-base metal complexes, specifically $Mn^{(II)}$ and $Co^{(II)}$, exhibit notable antioxidant capabilities that protect sperm DNA and may be utilized in in vitro sperm washing media. However, additional preclinical investigations are required to confirm their safety and efficacy.

1. Introduction

Male infertility is a major social and clinical problem. About 15% of couples have trouble getting pregnant, and male factors are to blame for about half of these cases. After standard tests, about 30% of men are considered idiopathic [1-3]. Oxidative stress (OS) is a fundamental mechanistic process resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defenses. While small amounts of reactive oxygen species (ROS) are necessary for capacitation and signaling, high levels of ROS cause sperm DNA fragmentation (SDF), which negatively impacts motility, fertilization, pregnancy rates, and embryo development [4-6].

To keep redox balance, you need natural antioxidant systems. Endogenous enzymatic defenses (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic scavengers (e.g., glutathione, vitamins C and E, coenzyme Q10, trace elements such as zinc and selenium) collectively mitigate reactive oxygen species (ROS); notably, infertile men often display reduced seminal antioxidant capacity [7-9]. Natural antioxidant supplementation, particularly multi-nutrient regimens and coenzyme Q10, has been clinically associated with improvements in semen quality and oxidative biomarkers; however, the effect sizes and methodologies differ significantly [10-14].

Nevertheless, the elevated expense and restricted availability of natural antioxidants promote the advancement of synthetic antioxidants that may provide equivalent protection to sperm DNA. Recent studies have confirmed the efficacy of synthetic antioxidants,

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particularly Schiff base ligands and their metal complexes, in neutralizing free radicals and preserving DNA integrity. Çolak et al. (2025) demonstrated substantial antioxidant activity of Co(II)/Pd(II) Schiff complexes [15], whereas Shafie (2025) brought out the redox properties of Schiff–Cu(II) systems [16], Ramadan et al. (2024) reported DNA-protective effects of mononuclear Schiff's base metal complexes [17], and Qamar et al. (2023) shone a spotlight on their part in preserving sperm DNA integrity [18]. Schiff-base scaffolds and their metal chelates provide tunable redox and coordination properties that may enhance radical scavenging and protect sperm genomic integrity [19-22].

Aim of the project: This research endeavor seeks to prepare and characterize novel laboratory-synthesized antioxidant compounds, with a keen spotlight on Schiff's base complexes, and to evaluate their promising role in warding off oxidative stress-induced sperm DNA fragmentation in human sperm.

2. Materials and Methods

2.1. Study design

This work included the synthesis of a singular Schiff base ligand and its metal complexes by the condensation (reflux) of a chosen aldehyde and primary amine. The resultant compounds were characterized by spectroscopic (FT-IR, UV-Vis, ^{1}H, ^{13}C-NMR) and analytical methods, including melting point, magnetic susceptibility, and conductivity assessments. The antioxidant efficacy of the synthesized compounds was evaluated by the DPPH radical scavenging test, and compounds exhibiting favorable IC_{50} values were chosen for further assessment. Oxidative stress was produced in human sperm using H_2O_2, and the protective properties of the chosen chemicals were evaluated using the Comet assay. Ascorbic acid served as the standard control. DNA damage was validated using gel electrophoresis and seen using a fluorescence microscope. Data were collected and subjected to statistical analysis to ascertain significance.

2.2. Synthesis of Schiff's base ligand [L] and complexes

The Schiff's base ligand [L], [NO_3] type [(S)-2,4-bis(((E)-4-(dimethylamino)benzylidene)amino)-4-oxobutanoic acid] was synthesized by reacting L-asparagine with 4-dimethylaminobenzaldehyde in a 1:2 molar ratio at 80°C for 8 h. The resulting precipitate was recrystallized at 4°C for 48 h. Metal complexes [Co_2(L).3Cl.H_2O] (1), [Mn_2(L).3Cl.H_2O] (2), and [Zn_2(L).3Cl.H_2O] (3) were synthesized by introducing metal chlorides ($CoCl_2·6H_2O$, $MnCl_2·4H_2O$, $ZnCl_2$) in a 2:1 ratio to the ligand under reflux for two hours. Products were subjected to filtration, ethanol washing, and subsequent drying (Scheme 1).

2.3. Characterization of the prepared compounds

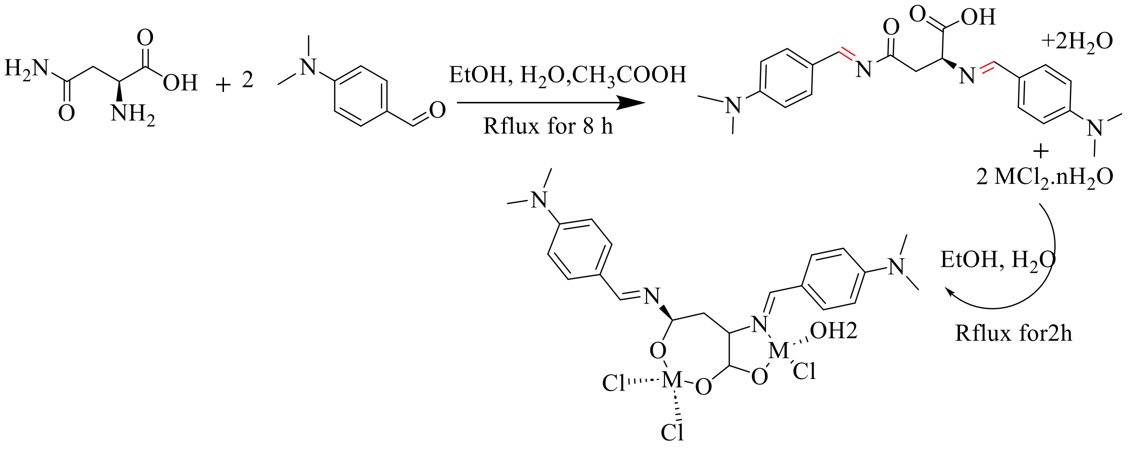
By using Fourier Transform Infrared Spectroscopy (Shimadzu IRTracer-100, Japan), the metal complexes and the synthesized ligand were analyzed. UV–Vis spectroscopy, Jasco V-730 spectrophotometer, Japan) NMR spectroscopy (^{1}H and ^{13}C), a Bruker Avance III 400 MHz spectrometer (Germany), Magnetic susceptibility, Conductivity measurements, WTW InoLab, Germany) These characterization techniques confirmed successful complexation and supported the proposed structural features of the synthesized compounds.

2.4. Antioxidant assay

The DPPH test assessed antioxidant activity. Compounds were solubilized in methanol at different doses (50–450 µg/mL). Absorbance was measured at 517 nm. IC_{50} values were determined by graphing percentage inhibition against concentration [23].

2.5. DNA protection assay

The Alkaline Comet assay (causing both double-stranded and single-stranded) was conducted to evaluate (prevention model) (H_2O_2-induced DNA damage prevention).



Scheme 1. Synthetic route of [L(1)] and its complexes. where M = Co$^{(II)}$, Mn$^{(II)}$, and Zn$^{(II)}$.

Semen was obtained from a single healthy volunteer whose sample met all standard reference criteria, including sperm concentration, motility, morphology, and semen volume. The use of a single donor was intentionally chosen to eliminate inter-individual biological variability and to ensure that all observed differences were attributable solely to the tested compounds rather than variations between different individuals.

Semen was washed once in PBS and adjusted to 1×106 cells/mL. Cells were co-incubated for ≥90 min at 37 °C with H_2O_2 (200 µM) alone or together with ascorbic acid (200 µM) or the test articles-[L] and its $Co^{(II)}$, $Mn^{(II)}$, $Zn^{(II)}$ complexes at 50 or 400 µg/mL. Three-layer agarose slides were cast on pre-warmed comet slides (base 0.5% NMA; cell layer 0.7% LMA with ~10^4 cells; top 0.7% LMA) and gelled at 4 °C. Slides were lysed for 2 h at 4 °C in 2.5 M NaCl/100 mM EDTA/10 mM Tris (with 10% DMSO), then alkaline-unwound 40–60 min in 300 mM NaOH/1 mM EDTA (pH>13) [56]. Electrophoresis was performed at 25 V, 300 mA, 30 min. After neutralization in 0.4 M Tris-HCl, pH 7.5 (3×10 min) and staining with ethidium bromide (60 µL, 10 min, dark), comets were imaged on a Leica DM4000 B LED (ex 515–560 nm).

For each condition, 100 cells/sample were scored. Primary endpoints were comet-head median (Min–Max) and %Head/%Tail DNA (mean ±SD); intensity-based metrics were intentionally omitted to avoid chromophore bias from colored metal complexes. The extent of sperm DNA fragmentation was quantified using CometScore (TriTek Corporation, Release 2.0.0.0, 2017), following the standard Comet Assay evaluation workflow [24-26].

2.6. Statistical analysis

Statistical analyses were performed using MedCalc Statistical Software, version 20 (MedCalc Software Ltd, Ostend, Belgium). Data distribution was assessed for normality using appropriate tests. Since the data did not follow a normal distribution, non-parametric tests were applied. Comparisons among groups were carried out using the Kruskal-Wallis test. For parameters showing significant overall differences, post hoc pairwise comparisons were conducted to identify specific group differences. Data are presented as median (interquartile range or minimum–maximum values, as applicable). A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Characterization

Comprehensive characterization data presented in the supplementary file confirm the successful synthesis of the Schiff's base ligand and the formation of its metal complexes.

3.2. Antioxidant activity

Vitamin C, a common antioxidant, had the highest radical scavenging activity of all the compounds tested, with a mean inhibition of 97.98 ± 0.23% and an IC$_{50}$ value of 21.7 µg/mL. This shows that it is very effective. The free ligand [L], on the other hand, had a much lower antioxidant efficiency, with only 50.21 ± 0.04% inhibition and a high IC$_{50}$ of 481.4 µg/mL. This suggests that it does not work well as a radical scavenger when it is not coordinated.

The $Mn^{(II)}$complex $[\mathrm{Mn_2(L).3Cl.H_2O}]$ exhibited a significant inhibition rate of 95.86 ± 0.27% and an IC$_{50}$ value of 54.7 µg/mL, closely aligning with that of AA. The $Co^{(II)}$)complex $[\mathrm{Co_2(L).3Cl.H_2O}]$ exhibited moderate activity, achieving 72.94 ± 0.42% inhibition and an IC$_{50}$ of 183.7 µg/mL. In contrast, the $Zn^{(II)}$complex $[\mathrm{Zn_2(L).3Cl.H_2O}]$ displayed relatively lower efficacy, with 63.60 ± 0.23% inhibition and an IC$_{50}$ value of 307.6 µg/mL (Table1).

3.3. DNA protection

Relative to H_2O_2, all L-based treatments improved DNA partitioning toward the head with concomitant reductions in tail DNA, indicating prevention of fragmentation. [L] (50 µg/mL) closely approached the ascorbic acid profile median (Min–Max) 88.37 (78.64–94.83), (head DNA, 92.12±7.10%; tail DNA, 7.87±7.10%), whereas [L] (400 µg/mL) provided a more moderate effect (head, 88.04±10.10%; tail, 11.95±10.50%).

Complexation enhanced protection: $[\mathrm{Co_2(L).3Cl.H_2O}]$and $[\mathrm{Mn_2(L).3Cl.H_2O}]$at 400 µg/mL achieved near-complete preservation (median 100.00 with head DNA, ≈99.9% and tail DNA, ≤0.12%), clearly outperforming H_2O_2 and effectively matching or exceeding ascorbic acid in DNA compartmentalization. In contrast, $[\mathrm{Zn_2(L).3Cl.H_2O}]$ afforded partial protection at both doses (head, ≈79–81%; tail, ≈19–21%). (Figure 1 and Table 2 and 3).

FT-IR spectra showed C=N stretches around 1635 cm^{-1} (Fig. 1-6 Supp. and Table 1 Supp.), shifting upon metal coordination[21, 27-29]. UV–Vis spectra exhibited ligand-centered (π→π*, n→π*) and metal-centered (d–d) transitions (Fig. 7-10 Supp. and Table 2 Supp.) [30-33]. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.72(S,1H), δ 8.74 (S,1H), δ 8.41(S,1H), 7.57 (d, J = 7.7 Hz, 2H), 6.72 (d, J = 7.7 Hz, 2H), 4.43 (t, J = 6.7 Hz, 1H), 3.27 – 3.19 (m, 1H), 3.02 – 2.94 (m, 1H), 2.19(S,12H),^{13}C NMR (125 MHz, DMSO-d_6) δ 177.87, 172.76, 162.72, 160.18, 152.17, 130.88, 130.64, 129.05, 127.18, 111.85, 70.53, 40.34, 40.03. (Fig. 11-12 Supp.) [34-36]. Magnetic susceptibility indicated high-spin tetrahedral geometries for $Co^{(II)}$ and $Mn^{(II)}$ complexes (Table 3 Supp.) [37-41] Conductivity values confirmed a 1:1 electrolytic nature (Table 4 Supp.).

Table 1. The IC_{50} and Mean Inhibition% ± SD Values of Tested Compounds Compared to Ascorbic Acid

Sample Name	C max (µg/mL)	Mean Inhibition ± SD	IC_{50}
Ascorbic Acid	60	97.98 ± 0.23%	21.7
[L1]	450	50.21 ± 0.04%	481.4
$[Co_2(L).3Cl.H_2O]$	450	72.94 ± 0.42%	183.7
$[Mn_2(L).3Cl.H_2O]$	450	95.86 ± 0.27%	54.7
$[Zn_2(L).3Cl.H_2O]$	450	63.60 ± 0.23%	307.6

Table 2. Comet assay results of ligand [L] and its complexes.

Group	Concentration (µg/mL)	Comet head Median (Min-Ma)	Head DNA (%) ±SD	Tail DNA (%) ±SD	Note
Intact sperm	–	82.81 (75.53-90.50)	82.65 ± 5.45	17.34 ± 5.45	Baseline
H_2O_2 (HP)	–	60.73 (56.58-77.09)	64.03 ± 8.18	35.97 ± 8.18	Damage control
Ascorbic acid	–	96.06(80.27-100.00)	93.64 ± 7.80	6.35 ± 7.80	Positive control
[L] (C1)	50	88.37 (78.64-94.83)	92.12 ± 7.10	7.87 ± 7.10	Strong protection
[L] (C1)	400	89.31(81.79-94.83)	88.04 ± 10.10	11.95 ± 10.50	Moderate < AA
$[Co_2(L).3Cl.H_2O]$(C2)	50	89.64 (87.42-91.80)	89.70 ± 1.50	10.29 ± 1.50	Clear protection
$[Co_2(L).3Cl.H_2O]$ (C2)	400	100.00 (99.73–100.00)	99.95 ± 0.10	0.05 ± 0.10	Near-complete prevention
$[Mn_2(L).3Cl.H_2O]$(C3)	50	100.00 (100.00-100.00)	99.95 ± 0.10	0.05 ± 0.10	Near-complete prevention
$[Mn_2(L).3Cl.H_2O]$(C3)	400	100.00 (100.00-100.00)	99.87 ± 0.30	0.12 ± 0.30	Near-complete prevention
$[Zn_2(L).3Cl.H_2O]$(C4)	50	77.04 (71.97-93.46)	80.88 ± 8.90	19.11 ± 8.90	Partial
$[Zn_2(L).3Cl.H_2O]$(C4)	400	77.90 (71.97-87.95)	78.91 ± 6.10	21.08 ± 6.10	Partial

Comet head = median (Min–Max), head/tail DNA = mean ± SD.

Table 3. Comet head values of the prepared compounds compared to ascorbic acid as a standard antioxidant

Compounds			Comet head value		Statistical analysis	
Mixture	Compound added	Concentration	Median	Min-Max	significance	P value
INTACT SPERM $_1$	-	-	82.81	75.53-90.50		
SPERM+HP ^{2}	-	-	60.73	56.58-77.09		
SPERM+HP+VIC^3	Ascorbic acid		96.06	80.27-100.00		
C1	Ligand	50 µg/mL	88.37	78.64-94.83	2	0.0001
C1	Ligand	400 µg/mL	89.31	81.79-94.83	1,2	0.00010
C2	$[Co_2(L).3Cl.H_2O]$	50 µg/mL	89.64	87.42-91.80	1,2,5	0.00008
C2	$[Co_2(L).3Cl.H_2O]$	400 µg/mL	100.00	99.73-100.00	1,2,3,4	0.00008
C3	$[Mn_2(L).3Cl.H_2O]$	50 µg/mL	100.00	100.00-100.00	1,2,3	0.00005
C3	$[Mn_2(L).3Cl.H_2O]$	400 µg/mL	100.00	100.00-100.00	1,2,3	0.00005
C4	$[Zn_2(L).3Cl.H_2O]$	50 µg/mL	77.04	71.97-93.46	2,3	0.00170

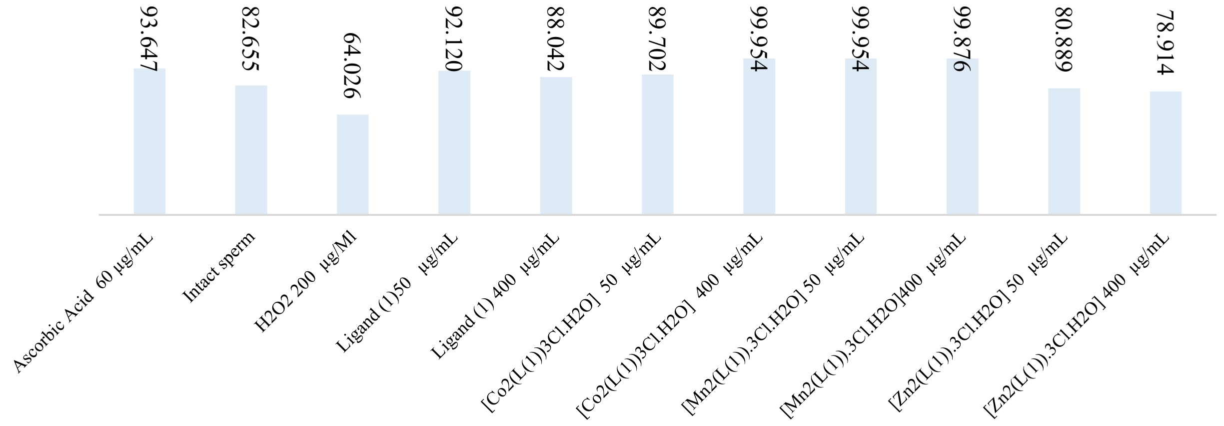


Fig. 1. Protective Effect of Synthesized Compounds on Head DNA Percentage under Oxidative Stress (Comet Assay)

The intimate electron transfer characteristics of the towered phenolic compounds could best explain the hierarchy in antioxidant ability among them. The strong radical-scavenging activity of AA (97.98 ± 0.23%) is attributed to its ability to donate hydrogen ions easily, thereby stabilizing the produced ascorbyl radical through resonance. By contrast, the free Schiff base ligand [L] showed only low antioxidant activity (50.21 ± 0.04%, the IC_{50} = 481.4 μg/mL) as there are no redox-active metal centers and limited π-electron delocalization present in this molecule. On the complexation with transition metal ions, a marked increase in antioxidant potential was noticed, especially for Mn(II) complex $[Mn_2(L).3Cl.H_2O]$ with 95.86 ± 0.27% inhibition and the IC_{50} 54.7 μg/mL, which was just about the same as that of ascorbic acid. The extraordinary enhancement can be attributed to the redox-active nature of $Mn^{(II)}$, facilitating catalytic electron transfer and the scavenging of reactive oxygen species (ROS).

The $Co^{(II)}$ complex exhibited moderate activity (72.94 ± 0.42%, IC_{50} =183.7 μg/mL), potentially due to Co(II) redox cycling inside the complex. The $Zn^{(II)}$ complex that did not react with redox had the lowest antioxidant activity (63.60 ± 0.23%, IC_{50} = 307.6 μg/mL). The markedly lower activity of the Zn(II) complex is expected, as Zn^{2+} is a redox-inactive metal ion with a stable d^{10} configuration. Unlike Mn^{2+}, which participates in redox cycling and ROS-scavenging pathways, Zn^{2+} cannot undergo electron-transfer reactions. This lack of redox reactivity explains its weaker antioxidant performance.

These results clearly show that the electronic structure and redox potential of the coordinated metal ion are what make an antioxidant strong (as shown in Table 1 and illustrated in Figure 1) [23, 42]. Comparable findings were reported by Çolak et al. (2025) [15] and Ramadan et al. (2024) [17], who showed that Schiff-base metal complexes with redox-active centers (Co, Mn, Cu) possess significantly enhanced radical-scavenging ability. Therefore, metal chelation not only stabilizes the ligand but also modulates its redox reactivity, thereby amplifying both its chemical and biological antioxidant capacity.

The Comet test was utilized to biologically confirm the antioxidant efficacy of these compounds. It demonstrated that the exposure of spermatozoa to hydrogen peroxide considerably elevated DNA fragmentation (head DNA = 64.03 ± 8.18%; tail DNA = 35.97 ± 8.18%). When treated with the free ligand [L], DNA damage decreased in a dose-dependent way. At 50 μg/mL, head DNA levels rose to 92.12 ± 7.10% and at 400 μg/mL, they rose to 88.04 ± 10.10%, which are similar to the levels of ascorbic acid (93.64 ± 7.80%). When Mn(II) and Co(II) were mixed, they preserved DNA almost completely (head DNA ≈ 99.9%, tail ≤ 0.12%, $P < 0.0001$). This was better than the free ligand and matched the ascorbic acid profile. On the other hand, the Zn(II) complex only gave partial protection (head ≈ 80–81%, tail ≈ 19–21%), which makes sense because it did not have a very strong DPPH scavenging activity (as shown in Figure 1 and Tables 2 and 3)[24, 25].

The close correlation between the chemical (IC_{50}) and biological (DNA integrity) findings emphasizes the crucial role of metal chelation in ROS detoxification and sperm genomic stabilization. These observations are consistent with those of Çolak et al. (2025) [15], who demonstrated the DNA-protective effect of $Co^{(II)}$ and $Pd^{(II)}$ Schiff-base complexes, as well as Qamar et al. (2023) [18], who reported that metal–ligand coordination significantly enhances sperm DNA resistance to oxidative insult.

Likewise, Ramadan et al. (2024) [17] described a dose-dependent DNA shielding effect in "$Fe^{(III)}$, $Ni^{(II)}$, and $Cu^{(II)}$ Schiff-base systems". In the present study, $Fe^{(III)}$, $Ni^{(II)}$, and $Cu^{(II)}$ complexes were not included due to their strong and often uncontrolled redox cycling, which can generate excessive ROS through Fenton-type reactions. These metals also carry higher risks of cytotoxicity and reproductive toxicity, making them unsuitable for a sperm-protection model. Therefore, our work focused on biologically safer ions-$Mn^{(II)}$, $Co^{(II)}$, and $Zn^{(II)}$-that are more compatible with reproductive applications.

Collectively, these findings indicate that the antioxidant and DNA-protective efficiencies of Schiff-base complexes are strongly governed by the redox activity of their central metal ions.

As a result, the synthesized $Mn^{(II)}$ and $Co^{(II)}$ complexes show a lot of promise as synthetic antioxidants that might be used to protect sperm and could be added to in vitro fertilization (IVF) conditions. However, more in vivo studies are needed to prove their safety, bioavailability, and compatibility with reproduction.

4. Conclusion

This study provides compelling evidence that Schiff-base metal complexes, especially $Mn^{(II)}$ and $Co^{(II)}$, serve as potent synthetic antioxidants. The $Mn^{(II)}$ combination exhibited an IC_{50} of 54.7 μg/mL while maintaining over 99.9% DNA integrity in sperm exposed to oxidative stress ($P < 0.0001$). The $Co^{(II)}$ complex exhibited similar protective properties. $Zn^{(II)}$ and the uncoordinated ligand showed some action. The findings suggest that Schiff-base complexes could function as efficacious treatments for oxidative stress-induced sperm DNA damage in vitro. More clinical tests are needed to find out how well they work as treatments, how well they are absorbed by the body, and how hazardous they are for use in living organisms.

Supplementary files

Supplementary file 1.

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