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Elevated seminal protein carbonyl concentration is correlated with asthenozoospermia and affects adversely the laboratory intracytoplasmic sperm injection (ICSI) outcomes

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Abstract

Elevated concentrations of reactive oxygen species (ROS) in the semen can lead to oxidative protein damage as they react with the amino acids' side chains in the protein, leading to the generation of carbonyl groups. This study aimed to investigate the effect of protein carbonyl (PC) concentration on sperm motility and the laboratory intracytoplasmic sperm injection (ICSI) outcomes. A total of 150 couples from the ICSI cycle were enrolled in this study and were divided into three groups (G) according to the PC concentration as following, G1 included samples with PC concentrations <0.65 nmol/mg, G2 included samples with 0.65≤PC≤2.23 nmol/mg and G3 included samples with PC>2.23 (nmol/mg). PC concentrations were measured in all semen samples, and the laboratory ICSI outcomes were evaluated for all injected oocytes. The Kruskal–Wallis *p*-values for the differences in the medians of sperm motility, fertilisation rate, embryo cleavage score and embryo quality score were <0.05. Furthermore, Dunn's post hoc test showed a significant difference between all groups, *p*-values <0.05, except for the medians of embryo quality score between G2 and G3. In conclusion, our results showed that sperm motility and laboratory ICSI outcomes are affected negatively by higher concentrations of PC in the semen.

KEYWORDS

embryo quality score, fertilisation rate, protein carbonyl, sperm motility

Abbreviations: DNP, 2, 4-dinitrophenyl; DNPH, 2, 4-dinitrophenylhydrazine; ELISA, Enzyme-linked immunosorbent assay; ICSI, Intracytoplasmic sperm injection; LDL, Low-density lipoprotein; NP, Nonprogressive motility; PC, protein carbonyl; PR, Progressive motility; PRBH, Prince Rashid Bin AL Hassan Hospital; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; UTI, Urinary tract infections.

1 | INTRODUCTION

Oxidative stress resulted because of an imbalance between reactive oxygen species (ROS) and antioxidants and characterised by an upset in the pro-oxidant–antioxidant balance in favour of the pro-oxidant, leading to oxidative damage (Sies, 1991). Antioxidants protect the cell from ROS by converting them into unreactive derivatives (Halliwell, 1996). Antioxidants control the levels of ROS without eliminating them, which would be highly expensive in terms of the energy required for the cell. Highly reactive ROS like hydroxyl radicals (OH) cannot be counteracted by antioxidants (Nathan, 2003). ROS play important roles in the cell as they play significant roles in regulating gene expression (Kunsch & Medford, 1999). Nitrogen oxide (NO) inhibits the oxidation of low-density lipoprotein (LDL) and prevents the aggregation of platelets (Hogg et al., 1993). Furthermore, low-level ROS functions as a physiological signalling messenger (Niki, 2016).

Measuring oxidative stress in humans requires accurate quantification of either free radicals or damaged biomolecules such as lipids, proteins and DNA (Morrow et al., 1999). Proteins are sensitive to ROS, leading to three types of modifications during oxidative protein damage: oxidation, glycation or glycoxidation (Berlett & Stadtman, 1997). Protein carbonyl is a biomarker of oxidative protein damage, induced as a result of ROS and reactive nitrogen species (RNS) exposure. ROS and RNS such as ONOO−, H_2O_2, NO• and •OH can react with several amino acids' side chains in lysine, arginine, proline and threonine, where the direct oxidation of these amino acids side chains lead to the formation of carbonyl derivatives (Len et al., 2019; Stadtman & Levine, 2000).

The level of protein oxidation depends on the type of oxidant and the protein structure. It is known that the hydroxyl radicals (OH−) are the most oxidising ROS, which itself is produced by radiolysis of H_2O or by Fenton reaction (Baraibar et al., 2013). Protein oxidative modifications affect protein stability and activity, as it may enhance the proteolysis or inhibit enzymatic action. The severity for such modification depends on the ratio of molecules that are altered (Oliver et al., 1987; Shacter, 2000).

Spermatozoa are susceptible to oxidative damage. Sperm's plasma membrane contains polyunsaturated fatty acids, where the peroxidation of these lipids produces secondary products that can oxidise sperm's proteins (Dalle-Donne et al., 2003). Spermatozoa with defective mitochondria generate a reduced amount of ATP and produce more ROS and free radicals, leading to further damage to mitochondria and mtDNA, which creates a state of energy imbalance and a reduction in sperm's motility (St. John et al., 2000). Abnormal spermatozoa are considered a major cause of endogenous ROS in the seminal fluid (Aitken et al., 2003). Furthermore, during urinary tract infections (UTI), leucocytes in the semen increase ROS levels one thousand times their levels in spermatozoa (Pasqualotto et al., 2000). Excess ROS is associated with poor semen quality like reduced sperm motility and abnormal sperm morphology (Iwasaki & Gagnon, 1992). ROS levels in the semen are negatively correlated with ICSI outcomes (Hammadeh et al., 2006).

Based on the aforementioned data, we hypothesised that protein carbonyl concentration in the semen of asthenozoospermic men is higher compared to the concentration in the semen of normozoospermic men. To test this hypothesis, we aimed to investigate the effect of protein carbonyl concentration on sperm motility, embryo cleavage score and embryo quality score. As far as we know this is the first study that investigates the effect of protein carbonyl concentrations on laboratory ICSI outcomes.

2 | MATERIALS AND METHODS

2.1 | Subjects

The samples were collected from 150 male partners who attended the ICSI cycle, at the IVF unit in Prince Rashid Bin AL Hassan Hospital (PRBH), Irbid, Jordan. Among them, 105 were asthenozoospermic males (progressive motility [PR] and nonprogressive motility [NP] <40%), while forty-five samples were collected from normozoospermic males with normal percentages of sperm motility, which ranged between 50% and 75% according to the 5th WHO edition (WHO, 2010). Samples were divided into three groups according to PC concentration as following: G1 included 44 samples with PC<0.65 nmol/mg, G2 included 86 samples with PC concentration between 0.65 and 2.23 nmol/mg and G3 included 20 samples with PC>2.23 nmol/mg. The cut-off point for each group was based on their Median ± *SD*. Patients with varicocele, alcoholic problems and cigarette smokers and patients with age above 40 were excluded from this study. Semen collection, sperm preparation for ICSI, oocyte denudation, the (ICSI) technique, fertilisation assessment, embryo grading, embryo cleavage score and embryo quality score were described previously (Al Smadi et al., 2021). This study was approved by the Jordanian Royal Medical Services-Human Research Ethics Committee on 30/7/2018 (TF3/1/Ethics Committee/9,126). Written consent from each couple was obtained.

2.2 | Fertilisation and embryo assessment

Oocytes were evaluated for fertilisation by the formation of two pronuclei after 16–18 hr post-ICSI. The fertilisation rate was calculated by dividing the number of fertilised oocytes by the total number of injected oocytes. On day 3, embryos were classified as grade 1 if they had 6–8 symmetrical blastomeres without fragmentation, grade 2 embryos had 6–8 symmetrical blastomeres with 10%–15% fragmentation, grade 3 embryos had <6 blastomeres, grade 4 embryos had >20% fragmentation or they had nonsymmetrical blastomeres, and grade 5 included arrested embryos or embryos totally fragmented (Scott et al., 2007). The embryo cleavage score was determined by counting the number of blastomeres in each embryo and given a number ranged from 1 to 4. The cumulative embryo cleavage score for each couple was determined by dividing the sum of all cleavage scores by the total number of

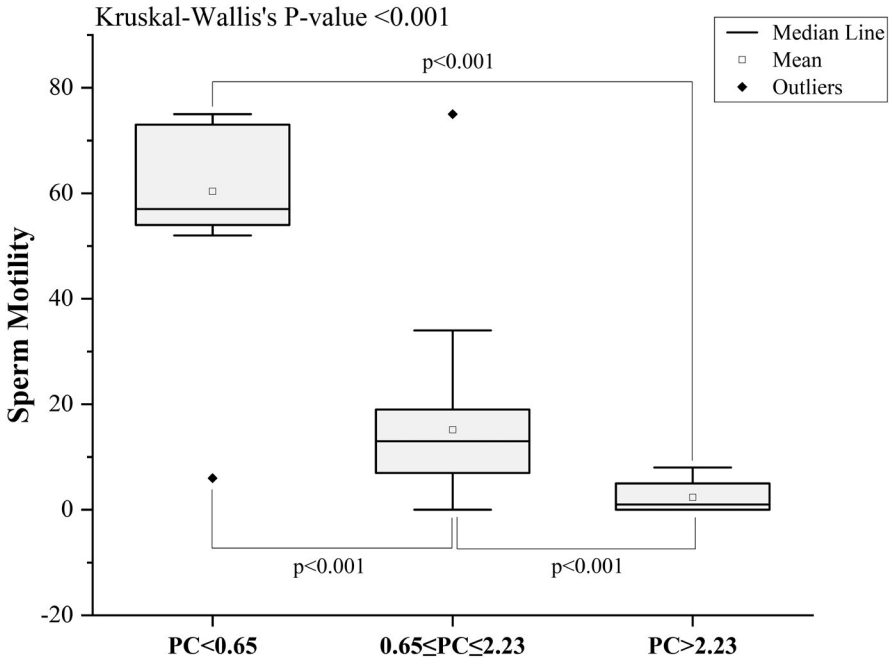

FIGURE 1 Box plots showing differences in the median sperm motility percentages between groups classified according to the protein carbonyl (PC) concentrations. Kruskal–Wallis *p*-values for the differences in the medians of sperm motility are <0.001. Dunn's post hoc *p*-values for the differences between groups are indicated

embryos, where embryos on day 3 with 8 cells were given 4 points, embryos with 6–7 cells were given 3 points, embryos with 4–5 cells were given 2 points and embryos with 2–3 cells were given 1 point. The embryo quality score was determined by examining the embryo morphology, and they were given a number ranged from 1 to 3. The cumulative embryo quality score was determined for each couple by dividing the sum of all embryo quality scores by the total number of embryos, where embryos with grade 1 were given 3 points, embryos with grade 2 were given 2 points, while embryos with grade 3, 4 and 5 were given only 1 point (Nasr-Esfahani et al., 2007).

2.3 | Protein isolation

Total protein was isolated from the semen using the Qproteome Mammalian Protein Prep Kit (Qiagen). The sperm count was adjusted to around 5 X 10^6 sperm/ml concentration, before starting the isolation, according to the kit's instructions. Total extracted protein was then stored at −20°C.

2.4 | Total protein quantitation

The protein concentration was measured by the Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific). The standard curve was prepared by plotting the average measurement at 595 nm for each bovine serum albumin (BSA) standard versus its concentration in μg/Ml. Then, the protein concentration for each unknown sample was determined using the standard curve. Protein samples were assayed in triplicates to confirm the accuracy of the measurements.

2.5 | Quantitation of protein carbonyl

Quantitation of protein carbonyl was performed using the OxiSelect™ Protein Carbonyl enzyme-linked immunosorbent assay (ELISA) Kit (Cell Biolabs). Total carbonyl groups were detected using this assay including derivatives of the protein carbonyl group and 2, 4-dinitrophenylhydrazine (DNPH), which in turn causes the production of a stable 2,4-dinitrophenyl (DNP). The total protein carbonyl was quantified using an ELISA microplate reader (Biotek) at 450 nm.

2.6 | Statistical analysis

Statistical analysis was carried out using the OriginPro, Version 2020 (OriginLab Corporation). The normality assumptions were checked for the variables in question and were found not to fit a normal distribution, and hence, nonparametric tests were applied. The Kruskal–Wallis H test was applied to decide whether the differences of variables between groups were significant, while Dunn's post hoc test was used to determine the differences between groups. A *p*-value <0.05 was considered to be statistically significant.

3 | RESULTS

3.1 | Effect of protein carbonyl on sperm motility

The median percentages for sperm motility among PC groups (G) were 57 ± 12.23 in G1, 13 ± 12.06 in G2 and 1 ± 2.72 in G3. These differences in sperm motility between groups were statistically

significant, *p*-values <0.05. Dunn's post hoc test was applied to determine which groups were different in sperm motility, and it showed a significant difference between all groups, *p*-values <0.05. (Figure 1). The sperm motility was higher among G1 which had low concentrations of PC compared to G3 which had high concentrations of PC (Table 1).

3.2 | Effect of protein carbonyl on fertilisation rate

The median fertilisation rates among the PC groups (G) were 67 ± 14.73 in G1, 40 ± 13.33 in G2 and 35.5 ± 7.72 in G3. These differences in fertilisation rates between groups were statistically significant, *p*-values <0.05. Dunn's post hoc test was applied to determine which groups were different in fertilisation rate, and it showed a significant difference between all groups, *p*-values <0.05. (Figure 2). The fertilisation rate was higher among G1 which had low concentrations of PC compared to G3 which had high concentrations of PC (Table 1).

3.3 | Effect of protein carbonyl on embryo cleavage score

The median embryo cleavage scores among the PC groups (G) were 3.66 ± 0.25 in G1, 3.34 ± 0.37 in G2 and 3 ± 0.32 in G3. These differences in embryo cleavage scores between groups were statistically significant, *p*-values <0.05. Dunn's post hoc test was applied to determine which groups were different in embryo cleavage scores, and it showed a significant difference between all groups, *p*-values <0.05 (Figure 3). The embryo cleavage score was higher among G1 which had low concentrations of PC compared to G3 which had high concentrations of PC (Table 1).

3.4 | Effect of protein carbonyl on the embryo quality score

The median embryo quality scores among the PC groups (G) were 2.5 ± 0.27 in G1, 1.82 ± 0.45 in G2 and 1.67 ± 0.30 in G3. These differences in embryo quality scores between groups were statistically significant, *p*-values <0.05. Dunn's post hoc test was applied to determine which groups were different embryo quality scores, and it showed a significant difference between G1 and G3, *p*-values <0.05, while G2 and G3 did not show a significant difference (Figure 4). The embryo quality score was higher among G1 which had low concentrations of PC compared to G3 which had high concentrations of PC (Table 1).

4 | DISCUSSION

Excess levels of ROS are associated with poor semen quality, manifested by reduced sperm motility and abnormal sperm morphology (Iwasaki & Gagnon, 1992). High ROS levels induce the peroxidation of polyunsaturated fatty acids leading to a reduction in sperm's motility. Protein carbonyl derivatives are produced relatively faster than other biomarkers and are relatively stable (Dalle-Donne et al., 2003).

In the present study, we found that sperm motility was negatively affected by protein carbonyl concentration. The highest percentages of sperm motility were among group one which included a lower concentration of protein carbonyl, while the lowest percentages of sperm motility were among group three which included a higher concentration of protein carbonyl. Our results agree with a previous study reported that high concentrations of protein carbonyl were associated with poor semen quality, and a negative correlation between protein carbonyl and sperm count (Shiva et al., 2011). The high amount of protein carbonyl was among infertile men with a sperm count of less than 10 million per millilitre, while it was low among normozoospermic men with sperm count higher than 80 million per millilitre (Shiva et al., 2011).

In this study, different laboratory ICSI outcomes including the fertilisation rate, the embryo cleavage score and the embryo quality score were found to be affected by the concentration of protein carbonyl. The fertilisation rate and the embryo cleavage score were higher among G1 and G2 compared to G3 which included a higher concentration of protein carbonyl with a significant difference between all groups, while the embryo quality score was higher among G1 and G2 compared to G3, with a significant difference between G1 and G3, while G2 and G3 did not show a significant difference. The absence of a significant difference between G2 and G3 groups could be explained by the sensitivity of embryo quality to higher concentrations of protein carbonyl. It seems that this

TABLE 1 Median sperm motility and laboratory ICSI outcomes among the studied groups

	Group 1 (*n* = 44)	Group 2 (*n* = 86)	Group 3 (*n* = 20)	*p*-value
Sperm motility %	57 ± 12.23	13 ± 12.06	1 ± 2.72	0.0001
Fertilisation rate %	67 ± 14.73	40 ± 13.33	35.5 ± 7.72	0.0001
Embryo cleavage score	3.66 ± 0.25	3.34 ± 0.37	3 ± 0.32	0.0001
Embryo quality score	2.5 ± 0.27	1.82 ± 0.45	1.67 ± 0.30	0.0001

Note: Group 1: included samples with PC concentrations <0.65 nmol/mg, Group 2 included samples with 0.65≤PC≤2.23 nmol/mg and Group 3 included samples with PC>2.23 (nmol/mg).
Abbreviations: ICSI, intracytoplasmic sperm injection; *n*, number; PC, protein carbonyl.

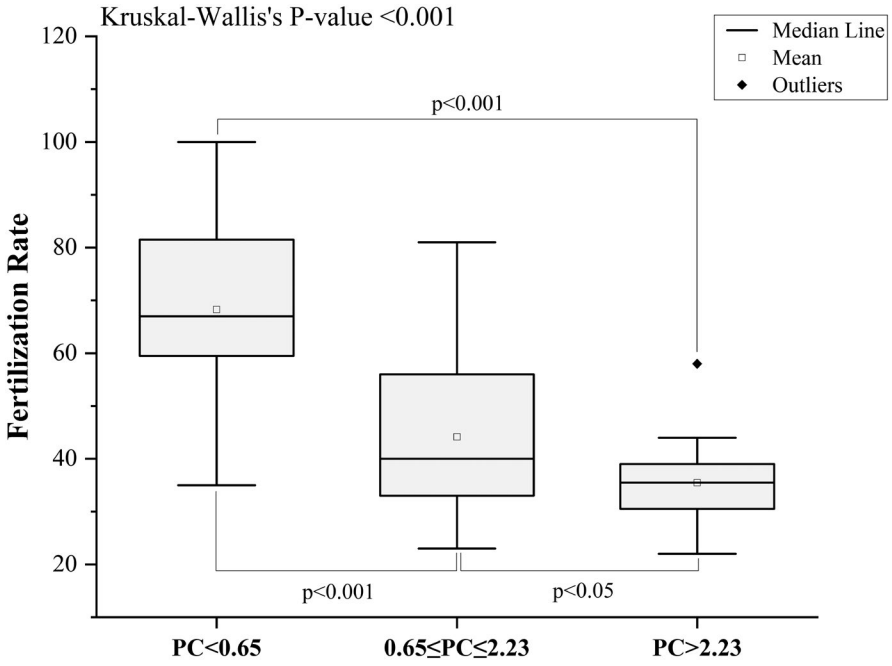

FIGURE 2 Box plots showing differences in the median fertilisation rates between groups classified according to the protein carbonyl (PC) concentrations. Kruskal–Wallis *p*-values for the differences in the medians of the fertilisation rate are <0.001. Dunn's post hoc *p*-values for the differences between groups are indicated

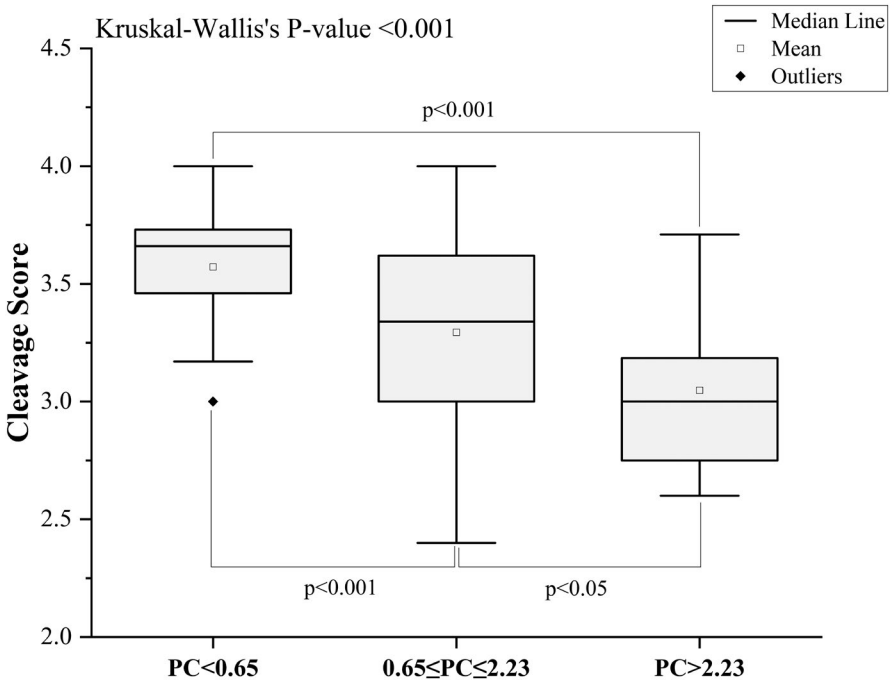

FIGURE 3 Box plots showing differences in median embryo cleavage scores between groups classified according to the protein carbonyl (PC) concentrations. Kruskal–Wallis *p*-values for the differences in the medians of the embryo cleavage score are <0.001. Dunn's post hoc *p*-values for the differences between groups are indicated

negative impact of protein carbonyl on embryo quality has a limit as a higher concentration of PC has no remarkable effect on embryo quality. Our results agree with a previous study that reported a negative correlation between ROS levels and the ICSI outcomes and found a negative correlation between ROSlevels and sperm quality (Hammadeh et al., 2006), while our results disagree with a previous study reported that ICSI has no clear advantage in males with normal semen parameters (Geng et al., 2020). Padron et al. demonstrated that ROSproduction is inversely correlated with sperm motility, and found elevated levels of ROSamong the majority of infertile males studied (Padron et al., 1997).

Protein carbonylation was also associated with sperm cryopreservation, where protein carbonyl levels were elevated by 20% in frozen bull spermatozoa, compared to fresh sperm samples (Mostek et al., 2017). Cryopreservation is thought to induce ROS generation, leading to affect sperm's proteins, DNA and lipids adversely (Agarwal et al., 2014). The freezing and thawing cycles also induce changes in the position of lipid membranes, which leads to an increase in the fluidity and calcium ions inside the cell (Cormier & Bailey, 2003).

Protein carbonyl was not linked before to sperm motility or ICSI outcomes, but several studies reported that it is associated with other diseases. Aksenov et al. found that protein carbonyl was

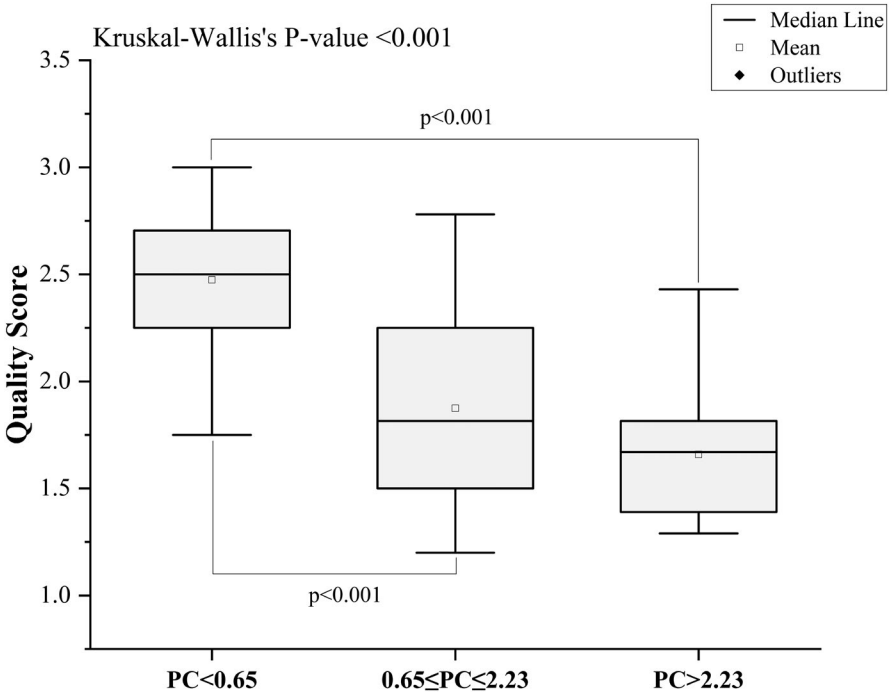

FIGURE 4 Box plots showing differences in the median embryo quality scores between groups classified according to the protein carbonyl (PC) concentrations. Kruskal–Wallis *p*-values for the differences in the medians of the embryo quality score are <0.001. Dunn's post hoc *p*-values for the differences between groups are indicated

positively correlated with Alzheimer's disease (AD), as protein carbonyl was elevated among AD patients compared to normal individuals (Aksenov et al., 2001). Another disease associated with protein carbonyl is coronary artery disease (CAD), as the protein carbonyl levels were found to be elevated among patients diagnosed with CAD, compared to normal individuals (Mutlu-Türkoğlu et al., 2005). Furthermore, the rare ophthalmic disorder, Eales disease, was found to be positively correlated with protein carbonyl levels and the content of protein carbonyl varied with the disease stages; therefore, protein carbonyl is a useful biomarker for disease severity (Rajesh et al., 2004). Finally, the hepatitis C virus (HCV) infection was found to be correlated with protein carbonyl, as the levels of protein carbonyl were elevated among patients infected with this virus; and an association between the alanine aminotransferase (ALT) enzyme—a marker of liver damage—and protein carbonyl was also established (De Maria et al., 1996).

How could ROS affect sperm motility? Mitochondrial dysfunction is caused by the generation of ROS at complex I in the inner membrane, thereby causing peroxidative damage to the midpiece of the spermatozoa, leading to a reduction in its motility (Koppers et al., 2008). ROS attacks different parts of the spermatozoa, including both mitochondrial and nuclear DNA (Agarwal et al., 2008). The absence of the cytoplasm in mature spermatozoa minimises the viability of antioxidants as the cytoplasm in the cell is considered as the main source of antioxidants and leading to a deficiency in the repair mechanism (Agarwal & Allamaneni, 2004).

In conclusion, our results showed that sperm motility, the fertilisation rate, the embryo cleavage score and the embryo quality score were affected negatively by the protein carbonyl concentrations. This demonstrates that protein carbonyl can be used as a biomarker for oxidative stress and can predict laboratory ICSI outcomes. Further studies are needed to explore how protein carbonylation affect embryo quality specifically at the ICSI settings.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MAS conducted the experiments and generated the manuscript. MEH designed the study and contributed to data interpretation and analysis. ESH served as clinical supervision. OB edited the manuscript. MMA involved in statistical analysis. MYJ designed the methodology (Bradford Assay). AM designed the methodology (ELISA). HA: involved in methodology and supervision. All authors approved the final version and the submission of this article.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATION

The study was approved by the Jordanian Royal Medical Services-Human Research Ethics Committee on 30/7/2018 with the project identification code (TF3/1/ Ethics Committee/9,126).

DATA AVAILABILITY STATEMENT

Data are available upon request from the corresponding author.

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