Changes in the Antioxidant Capacity and Iron-Binding Properties of Bovine Spermatozoa Following *In Vitro* Incubation with Ferrous or Ferric Iron

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Abstract

The aim of this study was to assess the impact of ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron on the antioxidant capacity and the ability to bind iron of bovine spermatozoa at specific time intervals (0h, 2h, 8h, 16h and 24h) during an *in vitro* culture. 35 semen samples were collected from 7 adult breeding bulls and diluted in physiological saline solution supplemented with different concentrations (0, 1, 5, 10, 50, 100, 200, 500, 1000 µmol/L) of $FeCl_2$ or $FeCl_3$. Spermatozoa motility was assessed using the SpermVision^{TM} CASA (Computer aided sperm analysis) system. The ferric reducing ability of plasma (FRAP) assay was applied to study the antioxidant capacity of the samples, while the ability of the sample to bind excess iron was determined using the Total iron-binding capacity (TIBC) test. Both ferrous and ferric iron exhibited a dose- and time-dependent impact on the spermatozoa motility. Concentrations ≥50 µmol/L $FeCl_2$ and ≥100 µmol/L $FeCl_3$ led to a significant decrease of spermatozoa motion ($P<0.001$), while concentrations below 10 µmol/L $FeCl_2$ and 50 µmol/L $FeCl_3$ proved to preserve the parameter ($P<0.001$). The FRAP assay revealed that both ferrous as well as ferric iron had a similar effect on the FRAP marker of the samples: high concentrations led to a dramatic and significant ($P<0.001$) increase of the parameter, followed by a notable decrease of the reducing ability in the subsequent time periods, whose intensity was dependent upon the time, oxidation state of iron, as well as the time of analysis. Furthermore, supplementation of $FeCl_2$ and $FeCl_3$ had an impact on the capacity of the sperm culture to bind free iron, reflected in a significant decrease of the parameter ($P<0.001$) early on (Time 2h) in case of high doses of both oxidative states of this biometal. In a direct comparison, ferrous iron has been shown to be more toxic than ferric iron. Results from this *in vitro* study show that high concentrations of both forms of iron are toxic, while their low concentrations may have spermatozoa activity-promoting properties. 50 µmol/L $FeCl_2$ and 100 µmol/L $FeCl_3$ could be regarded as critical *in vitro* concentrations of ferrous or ferric iron when it critically accumulates with toxic outcomes.

Keywords: iron, spermatozoa, bulls, motility, FRAP, TIBC

1. Introduction

Iron (Fe) is an essential trace metal, playing diverse roles in health and pathology [1]. Fe is directly or indirectly involved in the synthesis of bioactive macromolecules, cellular respiration, growth and differentiation [2], which are intricately related to sperm production, maturation and metabolism [3].

Fe is essential for a proper structure and function of the male reproductive system [4, 5] however it may become toxic if accumulated in large quantities [6], leading to metabolic alterations, defective spermatogenesis, decreased semen quality, [7, 8] all of which may result in a compromised fertility [6]. At the same time, it is known that Fe exhibits a dual activity on the oxidative balance of living systems. Catalase, a heme containing, hence Fe-dependent enzyme, is

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commonly found in the ejaculate [9], protecting spermatozoa against spontaneous free radical toxicity and lipid peroxidation [10, 11]. On the contrary, Fe overload may lead to testicular and epididymal oxidative stress (OS) and depletion of lipid-soluble antioxidants, accompanied by damage to the lipids, proteins and DNA, impaired spermtaogenesis and subsequent infertility [12-14]. Therefore, its two-sided properties related to male reproduction cannot be ignored.

Motility is crucial to provide an optimal passage of the spermatozoon through the female reproductive tract and penetration into the oocyte [15]. Numerous reports [5, 15-18] point out the importance of sperm motility assessment in order to determine the fertilization capacity of males, to discuss the impact of environmental factors on male fertility and to study the reproductive performance affected by diseases [19].

The chemical diversity of antioxidants is related to difficulties in the separation, detection or quantification of an individual antioxidant within a complex sample. Hence, the total antioxidant capacity is often useful to evaluate the general protective characteristics of the sample based on the interactions between individual antioxidant molecules [20]. Several assays have been proposed to estimate the antioxidant capacity in animal reproductive cells and tissues, however the 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) or ABTS-based colorimetric method and the ferric reducing antioxidant power (FRAP) are the most common methods applied in animal research [21].

The most effective way to estimate the presence of iron in a sample is related to the determination of the total iron binding capacity (TIBC), interpreted as the Fe concentration when transferrin is completely saturated. Transferrin is the primary transport protein for Fe and represents an essential iron pool. If due to genetic, lifestyle, and environmental factors transferrin is unable to effectively regulate the amount of iron in the body, this will accumulate to toxic levels, with a negative impact on spermatozoa production [22]. Simultaneously, transferrin can support the reactive oxygen species (ROS) generation [23, 24] as it may be oxidized by hydrogen peroxide to release free iron, generate ferryl-heme as well as more ROS [25]. Hence, this marker has a major significance to understand the iron metabolism in sickness or health.

In order to provide more information on the *in vitro* mechanisms of action of iron in the spermatozoon, this study was aimed to investigate the impact of iron in two oxidation states (divalent-ferrous and trivalent-ferric form) on the antioxidant capacity and iron-binding properties of bovine spermatozoa during specific time periods.

2. Materials and methods

Bovine semen samples aimed for the proposed experiments were obtained in quintiplicates from 7 adult Holstein Friesian breeding bulls (Slovak Biological Services, Nitra, Slovakia). The samples had to accomplish the basic criteria given for the corresponding breed. Semen was obtained on a regular collection schedule using an artificial vagina. After collecting, the samples were stored in the laboratory at room temperature (22–25 °C).

Each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9 % w/v, Bieffe Medical, Italia) containing different concentrations of ferrous ($FeCl_2$) or ferric ($FeCl_3$) iron (0, 1, 5, 10, 50, 100, 200, 500, 1000 μmol/L), and using a dilution ratio of 1:40. The samples were cultured in 96 well plates (MTP, Greiner, Germany) at 37 °C.

At cultivation times of 0h, 2h, 8h, 16h and 24h, the following tests were performed:

Spermatozoa motility (percentage of spermatozoa with a motility >5 μm/s; %; MOT) was examined with the help of the Computer-aided sperm analysis (CASA) system using the SpermVision^{TM} program (Minitube, Tiefenbach, Germany) and Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan). The samples were placed into the Makler Counting Chamber (depth 10 μm, 37°C; Sefi Medical Instruments, Haifa, Israel) and immediately assessed. At least 1000 cells were evaluated in each sample [17].

The Ferric reducing ability of plasma (FRAP) assessment followed the original procedure described by Benzie and Strain [26]. It is a simple test to determine the total antioxidant power, based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. The FRAP reagent contains 10 mmol/L TPTZ (2, 4,6- tripyridyl-s-triazine) solution in 40 mmol/L HCl (Centralchem, Bratislava, Slovak Republic) plus 5 mL of 20 mmol/L $FeCl_3$ (Centralchem, Bratislava,

Slovak Republic) and 50 mL 0.3 mol/L acetate buffer (pH=3.6; Centralchem, Bratislava, Slovak Republic). Aliquots of 100 µL sample were mixed with 3 mL FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm for 4 min using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA).

The ability of the sample to bind excess iron was determined using the Total iron-binding capacity (TIBC) commercial kit (PLIVA-Lachema, Brno, Czech Republic). The sample was saturated by ferric iron ions which were subsequently removed by adsorption on light magnesium carbonate. The content of iron in saturated serum was determined with a commercial reagent set for iron measurement (BLT Iron Liquid 200; PLIVA-Lachema, Brno, Czech Republic) using the Genesys 10 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA).

Protein concentration was assessed using the DiaSys Total Protein (DiaSys, Holzheim, Germany) commercial kit and the semi-automated clinical chemistry photometric analyzer Microlab 300 (Merck®, Darmstadt, Germany). This measurement is based on the Biuret method: Cu ions (in form of copper sulphate) react with proteins to form a violet blue color complex in alkaline solution, and the intensity of the color is directly proportional to the protein concentration when measured at 540 nm [27].

Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism program (version 3.02 for Windows; GraphPad Software, La Jolla California USA, www.graphpad.com). Descriptive statistical characteristics (mean, standard error) were evaluated at first. As we focused to study the impact of different divalent or trivalent iron concentrations on the spermatozoa activity (experimental groups) in comparison to the control at a specific timeframe, thus taking one factor into consideration, one-way ANOVA was used for specific statistical evaluations. The Dunnet's test was used as a follow up test to ANOVA, based on a comparison of every mean to a control mean, and computing a confidence interval for the difference between the two means. The level of significance was set at *** ($P<0.001$); ** ($P<0.01$); * ($P<0.05$).

3. Results and discussion

Iron (Fe) is a trace metal representing an important ecophysiological component of reproductive cells and tissues. In relatively small amounts, Fe is an essential cofactor for a broad array of bioactive molecules; however disturbances in its regulation, accompanied by its altered concentrations may have a negative impact on male reproductive physiology. Thus, Fe may exhibit dual roles in male reproduction based on its ability to either maintain the internal milieu as a micronutrient or to overturn this balance as a catalyst responsible for structural or functional alterations, often caused by or accompanied by oxidative stress [1].

The CASA analysis showed a time-dependent decrease of the motility in all assessed groups (Table 1). The highest motility was detected at Time 0h. Furthermore we observed that the presence of 1000 µmol/L $FeCl_2$ caused an instant and significant decrease of spermatozoa motility ($P<0.05$) when compared to the control. Toxic effects of high doses of both forms of iron grew evident at Time 2h, with a significant inhibition of the motion at 50-1000 µmol/L $FeCl_2$ ($P<0.001$ in groups supplemented with 100-1000 µmol/L $FeCl_2$ and $P<0.05$ with respect to 50 µmol/L $FeCl_2$) as well as at doses of 200-1000 µmol/L $FeCl_3$ ($P<0.01$ in case of 1000 µmol/L $FeCl_3$; $P<0.05$ in terms of 200 and 500 µmol/L; Table 1). The inhibiting effect related to high concentrations of ferrous as well as ferric iron turned to be more profound at Time 8h, with significant differences related to the supplementation of 50-1000 µmol/L $FeCl_2$ ($P<0.001$ in case of 100-1000 µmol/L $FeCl_2$; $P<0.05$ with respect to 50 µmol/L $FeCl_2$; Table 1) as well as 100-1000 µmol/L $FeCl_3$ ($P<0.001$ in terms of groups supplemented with 200-1000 µmol/L $FeCl_3$; $P<0.05$ accounting for 100 µmol/L $FeCl_3$; Table 1). The dual roles of iron on the spermatozoa motion became visible at Time 16h, with a significant ($P<0.001$) inhibition of MOT in case of $FeCl_2$ and $FeCl_3$ concentrations ranging between 100 and 1000 µmol/L while 1-10 µmol/L $FeCl_2$ together with 5-10 µmol/L $FeCl_3$ proved to have favorable effects on the spermatozoa motility ($P<0.001$ in relation to ferrous iron, and $P<0.01$ with respect to ferric iron). At the end of the *in vitro* culture, the lowest motility was recorded in both experimental groups supplemented with the highest doses of $FeCl_2$ and

$FeCl_3$, although 1000 µmol/L $FeCl_2$ exhibited a higher degree of inhibition on the spermatozoa motion performance in contrast with 1000 µmol/L $FeCl_3$. Compared to the control, significantly lower motility characteristics were recorded with respect to concentrations varying from 50 to 1000 µmol/L $FeCl_2$ ($P<0.001$ in case of 50-1000 µmol/L $FeCl_2$; $P<0.001$ with respect 100-1000 µmol/L $FeCl_2$) along with 100-1000 µmol/L $FeCl_3$ ($P<0.001$). At the same time, a significantly higher motility was found in the experimental groups administered with 1-10 µmol/L $FeCl_2$ ($P<0.001$), as well as with 5-10 µmol/L $FeCl_3$ ($P<0.01$; Table 1).

Table 1. Spermatozoa motility (MOT; %) in the presence of ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron in different time periods

	MOT 0h [%]		**MOT 2h [%]**		**MOT 8h [%]**		**MOT 16h [%]**		**MOT 24h [%]**	
	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$
Ctrl	80.80±1.11		74.21±2.45		70.03±2.83		53.53±2.66		48.31±3.56	
A	67.33± 2.55*	73.81± 1.95	21.57± 3.44***	54.77± 1.09**	17.08± 2.31***	33.97± 1.57***	9.01± 1.73***	14.55± 1.81***	4.99± 1.87***	9.07± 2.13***
B	73.22± 1.99	77.89± 2.03	29.54± 2.40***	65.01± 1.89*	24.99± 1.81***	45.05± 1.01***	16.07± 2.09***	23.02± 1.40***	14.01± 1.15***	15.71± 1.10***
C	74.22± 2.47	77.07± 1.48	41.01± 3.20***	62.92± 1.18*	37.05± 2.72***	48.45± 1.97***	27.99± 1.20***	35.44± 1.61***	25.14± 1.55***	18.38± 1.97***
D	76.77± 3.00	79.00± 2.15	45.97± 1.67***	74.00± 1.68	45.01± 1.37***	57.99± 1.48*	35.56± 1.18***	39.98± 1.74***	26.26± 1.42***	30.31± 1.96***
E	78.02± 2.56	77.87± 2.32	57.09± 1.96*	75.09± 1.44	50.90± 1.59*	67.24± 1.59	47.44± 1.22	51.02± 1.15	34.09± 1.77***	47.00± 1.02
F	81.35± 1.75	79.95± 2.23	77.03± 2.19	77.07± 1.28	77.01± 1.57	75.65± 1.46	71.59± 1.38***	65.44± 1.04**	70.03± 1.91***	56.07± 1.22***
G	82.71± 1.66	80.48± 2.06	76.07± 2.09	71.99± 1.78	73.97± 1.60	70.01± 1.76	74.89± 1.24***	62.77± 2.08**	72.87± 2.06***	57.31± 2.07***
H	77.05± 1.18	79.06± 1.62	78.02± 1.76	71.09± 1.59	73.06± 2.01	69.00± 1.05	75.03± 1.63***	52.52± 1.71	70.03± 2.05***	41.00± 1.00

X – Mean; S.E. – Standard Error.
* - $P<0.05$; ** - $P<0.01$; *** - $P<0.001$.
Ctrl – 0 µmol/L $FeCl_2$/$FeCl_3$, A – 1000 µmol/L $FeCl_2$/$FeCl_3$, B – 500 µmol/L $FeCl_2$/$FeCl_3$, C – 200 µmol/L $FeCl_2$/$FeCl_3$, D – 100 µmol/L $FeCl_2$/$FeCl_3$, E – 50 µmol/L $FeCl_2$/$FeCl_3$, F – 10 µmol/L $FeCl_2$/$FeCl_3$, G – 5 µmol/L $FeCl_2$/$FeCl_3$, H – 1 µmol/L $FeCl_2$/$FeCl_3$.

The gradual decrease of spermatozoa motility in the experimental groups supplemented with high Fe doses may be acknowledged to the oxidative stress to which spermatozoa are subjected during the *in vitro* cultivation [28-30]. ROS over-generated due to the presence of iron directly affect key enzymes of glycolysis, leading to a reduction of intracellular ATP levels accounting for the gradual loss of sperm motility in the wake of oxidative damage [31].

Our spectrophotometric analysis of the Ferric reducing ability of plasma as an alternative method to assess the antioxidant power showed an increasing trend of the marker over the course of the *in vitro* cultivation of the sperm cells, even in the absence of iron (Table 2). Both ferrous as well as ferric iron had a similar effect on the FRAP marker of the samples: high concentrations led to a dramatic and significant ($P<0.001$) increase of the parameter, followed by a notable decrease of the reducing ability in the following time periods. The intensity of these wave-like changes in the FRAP activity was dependent upon the time, oxidation state of iron, as well as the time of analysis. Generally, the biggest changes in the marker were observed at concentrations of 50-1000 µmol/L $FeCl_2$ ($P<0.001$) and 50-1000 µmol/L $FeCl_3$ ($P<0.001$). Another interesting observation was that the wave-like effects of ferrous iron became visible and significant already at Time 2h, a similar activity of ferric iron was observed later (Time 8h), although the intensity of the difference was similar to the Fe^{2+} experimental groups. Addition of $FeCl_2$ and $FeCl_3$ at concentrations ≤10 µmol/L resulted in an increase of the FRAP marker during early stages of the *in vitro* culture, however at a later point, their presence resulted in a slower FRAP increase when compared to the control (Table 2). A significantly lower FRAP activity, probably due to the presence of low Fe concentrations were significant ($P<0.001$) at Time 24h in case of 5-50 µmol/L $FeCl_2$ (Table 3), and at Times 8h-24h in case of 5 and 10 µmol/L $FeCl_3$ (Table 2).

Table 2. The effects of various doses of of ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron on the ferric reducing ability of plasma (FRAP) in different time periods

	FRAP 0h [µmol/g prot]		**FRAP 2h [µmol/g prot]**		**FRAP 8h [µmol/g prot]**		**FRAP 16h [µmol/g prot]**		**FRAP 24h [µmol/g prot]**	
	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$
Ctrl	217.20±14.17		257.00±9.24		266.95±9.72		266.50±26.40		347.10±22.36	
A	197.60± 9.07	212.10± 14.35	624.00± 9.38***	639.00± 38.10***	537.00± 9.21***	648.00± 35.45***	474.10± 7.89***	535.00± 37.90***	458.40± 8.69***	439.10± 25.50***
B	213.60± 8.01	221.00± 15.81	560.00± 9.68***	519.00± 18.70***	683.00± 13.92***	673.00± 43.40***	669.00± 12.26***	634.00± 25.65***	566.20± 9.80***	516.10± 14.14***
C	216.00± 10.74	219.90± 21.76	446.40± 9.42***	429.90± 17.32***	529.00± 8.58***	519.00± 16.14***	589.00± 13.66***	540.00± 37.43***	623.60± 11.22***	600.70± 21.23***
D	221.40± 12.40	222.30± 12.75	385.80± 11.20***	371.70± 22.17***	430.60± 13.45***	425.40± 18.37***	433.80± 9.90***	488.40± 27.87***	559.00± 9.39***	600.20± 37.91***
E	194.40± 14.72*	221.60± 18.15	365.40± 16.36***	310.40± 23.41***	358.00± 9.65***	290.70± 26.39	369.60± 13.34***	383.00± 35.11	429.40± 14.36**	419.10± 29.51**
F	213.00± 9.65	223.40± 15.87	250.40± 17.40	211.50± 30.29*	275.40± 9.58	204.60± 11.52*	268.00± 11.68	224.10± 17.82*	319.00± 11.26***	250.80± 14.50***
G	210.20± 8.84	225.50± 18.84	250.60± 13.99	208.10± 14.69*	266.80± 12.29	202.60± 18.70*	277.60± 15.71	224.60± 17.70*	305.40± 9.36***	234.20± 27.11***
H	196.80± 11.32	221.10± 17.35	256.40± 14.77	249.40± 39.75	256.00± 9.14	224.60± 27.43***	308.60± 15.26	280.10± 15.81	357.20± 9.95	315.10± 12.75

* - P<0.05; ** - P<0.01; *** - P<0.001.

Ctrl – 0 µmol/L $FeCl_2/FeCl_3$, A – 1000 µmol/L $FeCl_2/FeCl_3$, B – 500 µmol/L $FeCl_2/FeCl_3$, C – 200 µmol/L $FeCl_2/FeCl_3$, D – 100 µmol/L $FeCl_2/FeCl_3$, E – 50 µmol/L $FeCl_2/FeCl_3$, F – 10 µmol/L $FeCl_2/FeCl_3$, G – 5 µmol/L $FeCl_2/FeCl_3$, H – 1 µmol/L $FeCl_2/FeCl_3$.

Table 3. The effects of various doses of of ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron on the on the total iron binding capacity (TIBC) in different time periods

	TIBC 0h [µmol/g prot]		**TIBC 2h [µmol/g prot]**		**TIBC 8h [µmol/g prot]**		**TIBC 16h [µmol/g prot]**		**TIBC 24h [µmol/g prot]**	
	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$	$FeCl_2$	$FeCl_3$
Ctrl	25.09±4.47		22.09±6.05		21.00±4.09		20.99±3.26		18.31±3.55	
A	27.32± 2.76	23.17± 4.75	21.39± 2.81***	20.17± 3.82	16.18± 3.36***	16.31± 0.76	13.12± 3.38***	13.81± 2.08***	8.31± 2.39***	11.12± 2.54***
B	26.57± 2.49	25.77± 4.80	22.12± 2.73***	21.16± 2.26	18.57± 3.40***	16.40± 2.09	14.01± 4.08***	14.01± 3.47***	9.42± 3.77***	11.37± 3.53***
C	27.85± 2.49	22.19± 1.63	23.19± 2.81	21.50± 1.11	19.04± 2.14***	17.01± 1.61	15.70± 3.38***	18.01± 2.94	12.16± 2.21***	12.16± 3.39***
D	26.55± 2.02	23.20± 2.39	23.29± 2.82	21.04± 2.61	19.29± 4.20***	18.13± 3.17	16.46± 4.37***	16.36± 3.07	13.60± 3.83***	14.04± 1.41
E	27.95± 2.04	22.18± 3.22	24.34± 3.44	21.19± 1.82	20.19± 3.26***	21.02± 3.60	16.09± 4.07***	18.01± 3.21	15.26± 2.56***	16.30± 1.26
F	28.47± 2.45	22.13± 2.70	25.29± 2.53	20.17± 1.62	23.37± 3.46	20.13± 3.20	21.46± 3.44	18.20± 3.19	20.53± 3.38	17.34± 2.17
G	26.33± 2.57	23.04± 2.55	25.43± 3.44	21.20± 2.27	23.37± 3.38	21.01± 2.21	20.29± 2.74	19.21± 1.48	20.43± 2.33	18.00± 1.58
H	26.26± 2.64	22.13± 2.21	24.85± 2.62	20.11± 3.81	22.11± 4.08	19.36± 3.50	20.15± 3.23	18.31± 2.19	19.68± 3.55	17.00± 2.35

X – Mean; S.E. – Standard Error.

* - P<0.05; ** - P<0.01; *** - P<0.001.

Ctrl – 0 µmol/L $FeCl_2/FeCl_3$, A – 1000 µmol/L $FeCl_2/FeCl_3$, B – 500 µmol/L $FeCl_2/FeCl_3$, C – 200 µmol/L $FeCl_2/FeCl_3$, D – 100 µmol/L $FeCl_2/FeCl_3$, E – 50 µmol/L $FeCl_2/FeCl_3$, F – 10 µmol/L $FeCl_2/FeCl_3$, G – 5 µmol/L $FeCl_2/FeCl_3$, H – 1 µmol/L $FeCl_2/FeCl_3$.

It has been reported on different occasions that a proper ROS detoxification, thus the balance between individual components of the antioxidant system in semen is important to maintain the spermatozoa motility [32-35]. Positive correlations between enzymatic or non-enzymatic antioxidants and sperm motility have been related to lower oxidative insults and cytotoxicity to spermatozoa. Measurements of antioxidant components and characteristics have also shown significant differences between case and control groups and correlated with sperm motility [32, 36].

Different authors noted a negative impact of iron on the characteristics of a variety of antioxidants, including alpha-tocopherol, ubiquinol and antioxidant proteins in the testes. Moreover, oxidative products were significantly higher in rats supplemented with high doses of iron in the experiments by Doreswamy and Muralidhara [37]. Previous studies have pointed out the cytosolic system as one of the major defense mechanisms of the cell against oxidative insults, based on its ability to specifically recognize and degrade oxidized by-products [37, 38]. Various *in vitro* studies using several oxidized substrates as a reference have demonstrated the degradation of antioxidant molecules [38, 40]. Such studies have used a variety of oxidizing agents, including iron [38, 40] as a source of oxidative damage [41]. Failure of the degradation of oxidized by- and end-products has been proposed as one of the major events promoting the disease-dependent accumulation of intracellular necrotic material [39]. Therefore, the degradation of oxidatively damaged molecules seems to be the major strategy of mammalian cells to deal with oxidized and inactivated biomolecules.

As seen in Table 3, supplementation of ferrous and ferric iron had an impact on the capacity of the sperm culture to bind free iron. Comparing ferrous and ferric iron, it is clear that the ferrous iron had a more serious and significant impact on the TIBC marker. $FeCl_2$ ≥500 µmol/L caused a significant decrease of the binding capacity ($P<0.001$) already at 2h, which in the case of $FeCl_3$ became notable at 16h. The impact of ferrous iron remained significant for the rest of the experiment and became more profound, as starting at 8h, a significantly decreased TIBC became associated with Fe^{2+} ≤500 µmol/L (50-200 µmol/L $FeCl_2$) as well (Table 3). In the case of ferric iron, the significant ($P<0.001$) TIBC reduction remained notable at 24h as well, although the concentration range related to a negative impact on the parameter was narrower (200-1000 µmol/L $FeCl_3$; Table 3). Other concentrations of $FeCl_2$ or $FeCl_3$ had no significant effect on the TIBC ($P>0.05$), and were not able to prevent the natural decrease of the parameter over the course of the *in vitro* culture.

Because cells are exposed to oxidative stress continuously or at least most of the time, investigating the fate of the protein bound active compounds seems important. This is especially true for iron because catalytically active free iron may enhance the oxidative damage of the cell. Transferrin is the major iron transport protein of the cell and is readily oxidized by hydrogen peroxide with an accompanying increase in proteolytic susceptibility [38].

The availability of transferrin as shown by the TIBC assay shows a fast decline of the reducing milieu of the samples supplemented with high Fe concentrations. It is discussable whether the TIBC marker declines based on the availability of free biding areas of the transferrin molecule of by the oxidative damage to the protein caused by excessive iron content and a subsequent rise of the hydroxyl radical.

Wise et al. [3] showed that the Fe amount was negatively correlated with the transferrin or ferritin availability and testicular weight in boars. Furthermore, boars with high Fe levels and low transferrin/ferritin produced less sperm. As the testicular Fe concentration increased, daily sperm production (DSP) and total DSP declined. The study concluded that abnormal activity of both transferrin and ferritin were associated with hypogonadism and Fe accumulation may lead to reduced sperm production.

4. Conclusions

Results from this *in vitro* report show that iron has time- and dose-dependent effects on the spermatozoon. High concentrations of both forms are toxic, resulting in a notable decrease of the spermatozoa activity, accompanied by a disturbance in the oxidative balance. The ability of the cell to process free iron was severely impaired too. At the same time, our data show that ferrous iron is more toxic and displays its deleterious

effects in a shorter period of time. It may therefore be suggested that while it takes more time for the Fe^{3+} to be reduced, Fe^{2+} is readily available for oxidative processes. Furthermore, as ferric iron is considered to be a physiological ion for the organism, the spermatozoon appears to be able to endure a higher load of $FeCl_3$ when compared to $FeCl_2$. Furthermore, concentrations below 10 µmol/L$FeCl_2$ and 50 µmol/L $FeCl_3$ proved to be stimulating to the spermatozoa physiology with very similar effects, and independently on the oxidative state of iron. Finally, we may hypothesize that 50 µmol/L $FeCl_2$ and 100 µmol/L $FeCl_3$ could be regarded as critical *in vitro* concentrations of ferrous or ferric iron when it ceases to be an essential micronutrient in order to become a serious toxic and pro-oxidant substance.

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