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Ferritin Overload Suppresses Male Fertility Via altered Acrosome Reaction

Woo-Sung Kwon, Md Saidur Rahman, Ye-Ji Kim, Do-Yeol Ryu, Amena Kahtun and Myung-Geol Pang†

Department of Animal Science and Technology, Chung-Ang University, Anseong 456-756, Korea

ABSTRACT

Iron is required for cell viability but is toxic in excess. While the iron-mediated malfunction of testicular cells is well appreciated, the underlying mechanism(s) of this effect and its relationship with fertility are poorly understood. Ferritin is a ubiquitous intracellular protein that controls iron storage, ferroxidase activity, immune response, and stress response in cells. Ferritin light chain protein (FTL) is the light subunit of the Ferritin. Previously, we had identified the FTL in bovine spermatozoa following capacitation. In present study, to investigate the role of Ferritin in sperm function, mice spermatozoa were incubated with multiple doses (1, 10 and 100 μM) of sodium nitroprusside (SNP), an iron donor. SNP was increased Ferritin levels in a dose-dependent manner. The Ferritin was detected on the acrosome in spermatozoa by immunocytochemistry. Short-term exposure of spermatozoa to SNP increased tyrosine phosphorylation and the acrosome reaction (AR). Finally, SNP affected a significant decrease in the rate of fertilization as well as blastocyst formation during early embryonic development. On the basis of these results, we propose that the effects of Ferritin on the AR may reduce overall sperm function leads to poor fertility in males and compromised embryonic development.

(Key words : Ferritin, Sodium nitroprusside, Acrosome reaction, Tyrosine phosphorylation, Fertility)

INTRODUCTION

While ample clinical and experimental data support the critical role that iron plays in all living organisms, it is also known to be toxic in excess. Iron homeostasis in cells is regulated by the highly conserved Ferritin protein through interactions with the iron regulatory protein (IRP) and the iron regulatory element (IRE) system (Kim and Ponka, 2002; Comporti, 2002). It is not known, however, whether Ferritin plays an active role in the mechanism of intracellular iron homeostasis in spermatozoa (Comporti, 2002). Iron overload has been implicated in the etiology of several disease conditions, such as hemochromatosis, adult-onset Still's disease, porphyria, hemophagocytic lymphohistiocytosis and β-thalassemia. Given these associations, a greater understanding of iron homeostasis would offer significant insights into the pathophysiology of numerous iron-mediated disorders.

In the male reproductive system, iron overload can be harmful, whereas depletion can reverse this pathology (Rajesh Kumar *et al*., 2002). Wellejus *et al*. (2000) reported that the administration of iron to rat testis results in testicular atrophy, morphological changes in the testes, impaired spermatogenesis, epididymal lesions and overall defective reproductive performance. Although it has been speculated that iron-mediated oxidation of testicular cells may be involved in triggering these changes in testicular morphology and function (Lucesoli *et al*., 1999), the underlying mechanisms remain largely unclear.

Puntarulo (2005) reported that iron-induced lipid peroxidation, expression of protein carbonyl and depletion of lipid soluble antioxidants in testicular tissue results in disturbances of overall spermatogenesis. More recently, it has been postulated that iron is involved in the generation of hydroxyl radicals via Fenton chemistry, which is believed to give rise to harmful reactive oxygen species (ROS) that are associated with oxidative damage to sperm DNA (Borkowska *et al*., 2011). This potential effect of excess iron is particularly important since lesions in sperm DNA may be transmitted to oocytes during fertilization, resulting in genotoxic effects in the zygote and embryo (Borkowska *et al*., 2011).

Wise *et al*. (2003) postulated an inverse relationship

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† Corresponding author : Phone: +82-31-670-4841, E-mail: mgpang@cau.ac.kr

in boars between concentrations of iron and Ferritin protein and testis size and weight. Moreover they demonstrated an adverse effect of increasing testicular iron concentrations on sperm production. Interestingly, it has been shown that human males with β-thalassaemia major have decreased pituitary function resulting from iron overload, whereas serum Ferritin is highly correlated with hypogonadism (Perera *et al*., 2002). Although the effect of disruption of iron homeostasis on male fertility has been the subject of research for several decades (Wise *et al*., 2003; Kamboj and Kar, 1964; Kim *et al*., 2006), several fundamental questions remain unanswered. For example, how does iron affect processes in spermatozoa, including capacitation, the acrosome reaction (AR) and fertilization? Moreover, what is the role of Ferritin when spermatozoa are exposed to conditions of iron overload?

To answer these questions, first we investigated the effect on mouse spermatozoa of the nitric oxide donor, sodium nitroprusside (SNP) [$Na_2(Fe(CN)_5NO)$], a potent *in vitro* iron inducer (Kim *et al*., 2006) and Ferritin biosynthesis (Kim and Ponka, 2002; Recalcati *et al*., 1998; Harrison and Arosio, 1996). Second, to clarify the exact role of iron in specific sperm fuction, we analyzed the effect of SNP on various sperm parameters related to capacitation and the AR, tyrosine phosphorylation (TYP). Moreover, to identify the potential role of iron in modulating fertility, the effect of SNP on fertilization and embryogenesis was assayed using *in vitro* fertilization (IVF).

MATERIALS AND MATHODS

Ethical Statement

All procedures with animals were performed according to guidelines for the ethical treatment of animals and permitted by Institutional Animal Care and Use Committee of Chung-Ang University, Seoul, Republic of Korea.

Media and Chemicals

Unless otherwise stated, all components were purchased from Sigma-Aldrich (St Louis, MO, USA). Modified Tyrode's medium was used as basic media (BM) and was prepared fresh before each experiment (Kwon *et al*., 2013a and b). Briefly, BM contain 97.84 mM NaCl, 1.42 mM KCl, 0.47 mM $MgCl_2 \cdot H_2O$, 0.36 mM $NaH_2PO_4 \cdot H_2O$, 5.56 mM D-glucose, 25 mM $NaHCO_3$, 1.78 mM $CaCl_2 \cdot H_2O$, 24.9 mM Na-lactate, 0.47 mM Na-pyruvateand 50 μg/mL gentamycin. The medium was pre-incubated one day prior to the experiment and bovine serum albumin (BSA; 4 mg/mL) was added to induce capacitation of spermatozoa. A stock solution of SNP was diluted with distilled water and stored in aluminum foil wrapped plastic container at 4℃ in the refrigerator dark chamber. Experiments using SNP were performed in presence of red light in a safety cabinet. Stock solution was diluted with BM to reach the desired final molar concentrations of 1, 10 and 100 μM.

Preparation and Treatment of Mouse Spermatozoa

Experimental mice were housed in a temperature (22±2℃), ventilation and light-controlled (12 h light/12 h dark) room and were provided with laboratory feed (Cargill Agripurina, Seongnom, Korea) with water *ad libitum*. The mouse sperm suspension was prepared using ICR 8～12 week-old male mice (Nara Biotech, Seoul, Korea). A standard procedure was followed to collect spermatozoa from the male mice (Kwon *et al*., 2013a and b; Lee *et al*., 2015; Kim *et al*., 2015). In brief, both cauda epididymis from each mouse were separated and associated fat was removed. The sample was placed on a piece of filter paper to remove the excess liquid. The cauda epididymis was punctured using a sterile needle in BM containing 0.4% BSA in cell culture dishes to facilitate the release of spermatozoa. The resulting spermatozoa were then incubated for approximately 10 min in the presence of 5% CO_2 at 37℃ to facilitate dispersal. Finally, the sperm suspension was incubated for further 90 min at same incubation condition in air for capacitation in BM supplemented with 1, 10 and 100 μM of SNP in separate falcon tube.

Western Blot Analysis for Detection of Sperm Proteins

Western blot analysis for Ferritin levels and tyrosine phosphorylation in mouse spermatozoa was carried out according to a previously described method (Rahman *et al*., 2014 and 2015). Briefly, each sample was washed twice with DPBS and centrifugation was carried out at 10,000 ×g for 10 min after treatment. Sperm pellets was resuspended in Laemmli sample buffer (63 mM 1 Tris, 10% glycerol, 10% sodium dodecyl sulfate, 5% bromophenol blue) containing 5% 2-mercaptoethanol and incubated for 10 min at RT. Finally, supernatants were separated by centrifugation at 10,000 ×g for 10 min. Samples were then subjected to SDS-PAGE using a 12 % mini-gel system (Amersham, Piscataway, NJ, USA) and separated proteins were transferred to a polyvinylidene fluoride membrane (Amersham). 3% blocking agent (Amersham) was employed to block the membrane for 1 h at RT. To detect Ferritin in SNP-treated spermatozoa, samples were incubated with anti-Ferritin light chain antibody (Abcam) diluted (1 μg/mL) with 3 % blocking agent overnight at 4℃. Horse-radish peroxi-

dase (HRP) conjugated polyclonal goat anti-rabbit IgG (Abcam) diluted with 3% blocking solution at a ratio of 1:5000 was then used to treat the membrane for 1 h at RT. Tyrosine phosphorylation was detected using a HRP-conjugated mouse monoclonal anti-phosphotyrosine antibody (PY20; Abcam) diluted with blocking solution (1:2,500) overnight at 4℃. The membrane was then incubated with an HRP-conjugated Goat Anti-Rabbit IgG (Abcam) diluted with blocking solution (1: 5,000) for 1 h at RT. α-tubulin was detected as an internal control using a monoclonal anti α-tubulin mouse antibody (Abcam) diluted with blocking solution (1:10,000) for 2 h at RT. Finally, the membranes were washed three times with PBST. Visualization of proteins on the membrane was performed with an enhanced chemiluminescence (ECL) technique using ECL reagents. The bands were scanned using a GS-800 calibrated imaging densitometer (Bio-Rad, Hercules, CA) and analyzed with Quantity One software (Bio-Rad). Finally, bands were calculated by the ratio of Ferritin/α-tubulin and phosphotyrosine/α-tubulin.

Combined Hoechst 33258/chlortetracycline Fluorescence Assessment of Capacitation Status (H33258/CTC)

The capacitation status of spermatozoa was determined by the dual staining method (Kwon *et al*., 2013a and b; Kwon *et al*., 2015). Briefly, 135 μL of treated spermatozoa were added to 15 μL of H33258 solution (10 μg H33258/mL DPBS) and incubated for 2 min at RT. Excess dye in the sperm sample was removed by layering the mixture over 250 μL of 2% (w/v) polyvinylpyrrolidone in Dulbecco's phosphate-buffered saline (DPBS). After centrifugation at 100 × *g* for 2.5 min, the excess supernatant was removed and the pellet was resuspended in 100 μL of DPBS; 100 μL of a freshly prepared chlortetracycline fluorescence (CTC) solution (750 mM CTC in 5 μL buffer: 20 mM Tris, 130 mM NaCl, and 5 mM cysteine, pH 7.4). Samples were observed by a Microphot-FXA microscope (Nikon, Tokyo, Japan) under epi-fluorescence illumination using ultraviolet BP 340-380/LP 425 and BP 450-490/LP 515 excitation/emission filters for H33258 and CTC, respectively. Finally, the spermatozoa were classified as live non-capacitated (F pattern), live capacitated (B pattern) and live acrosome-reacted (AR pattern) according to Maxwell and Johnson (1999). Two slides per sample were evaluated with at least 400 spermatozoa in each slide.

Localization of Ferritin in Spermatozoa

Immunolocalization of Ferritin was done following the standard method described previously (Kwon *et al*., 2013b and 2014a). Briefly, the spermatozoa released from the cauda epididymis were suspended in DPBS, placed on a glass slide, and allow to air dry. Paraformaldehyde (3.7%) was used to fix the sperm for 30 min at 4℃. DPBS containing 0.1% Tween-20 (PBST) was used to wash the cells and blocked with 5% BSA in PBST for 1 h at 4℃. The slide was then incubated with anti-Ferritin light chain antibody (Abcam, Cambridge, UK) diluted with 5% BSA in PBST (1:50), lectin peanut agglutinin (PNA), conjugated with Alexa Fluor 647 (Molecular Probes, Carlsbad, CA) and diluted with 5% BSA in PBST (1:100) overnight at 4℃. After 2 × washing of the slides in PBST and DPBS, slides were treated with fluorescein isothiocynate (FITC)-conjugated goat polyclonal rabbit IgG (Abcam) diluted with 5% BSA in PBST for 2 h at RT. Counterstaining was done with 4',6-diamidino-2-phenylindole (DAPI) and results were visualized using a TS-1000 microscope equipped with NIS elements image software (Nikon, Tokyo, Japan).

***In Vitro* Fertilization**

Eight-twelve weeks old B6D2F1/CrljOri hybrid female mice were purchased from Nara Biotech and superovulated by intra peritoneal injection of 5 IU of pregnant mare serum gonadotrophin and 5 IU of human chorionic gonadotrophin (hCG) 48 h later. Cumulus-oocyte complexes (COCs) were collected from oviducts in DPBS after 15 h hCG treatment. COCs were placed in 50 μL BM supplemented with 10% FBS under mineral oil and then incubated at 37℃ in 5% CO_2 in air for 90 min before insemination. After capacitation, treated spermatozoa were washed with BM supplemented with 0.4% BSA and 1×10^6 /mL spermatozoa were gently inseminated into the incubated COCs and further incubated for 6 h at 37℃ in the same incubation conditions. After fertilization normal zygotes were collected then incubated in 50 μL of BM supplemented with 0.4% BSA. 18 h after insemination, the fertilization rate was assessed by determining the number of two-cell embryos. All two-cell embryos were separated and cultured in a 50 μL volume of BM supplemented with 0.4% BSA for 4 days at 37℃ under 5% CO_2. All embryos were counted to the development of the blastocyst stage.

Statistical Analysis

Data were analyzed using a one-way ANOVA implemented in SPSS (Version 12.0, USA), and a Tukey's test was used to analyze differences among replicates. All tests were interpreted at a confidence level of 95% ($p<0.05$) and numerical data were represented as mean ±SEM.

RESULTS

Effect of SNP on Ferritin Levels in Spermatozoa

To measure the effect of SNP treatment on Ferritin levels in spermatozoa, we carried out Western blot analysis using a specific anti-Ferritin antibody. Fig. 1 shows a significant dose-dependent induction by SNP of Ferritin levels in spermatozoa. Additionally, subcellular localization of Ferritin in mouse spermatozoa was evaluated by immunofluorescence technique. The Ferritin protein was detected in the acrosome of the mouse spermatozoa (Fig. 2).

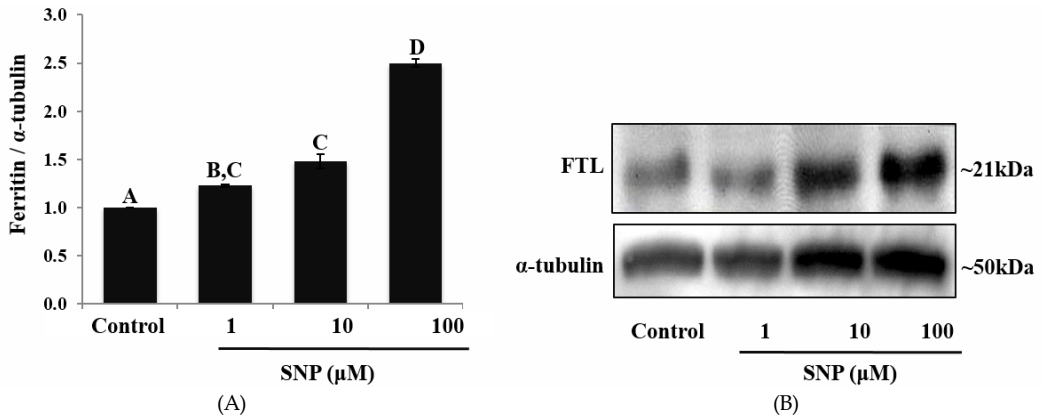


Fig. 1. Effect of SNP on Ferritin levels in spermatozoa. (A) Density of Ferritin (~21 kDa) in spermatozoa following incubation with different concentrations of SNP. Data represent the mean of 4 replicates±SEM. Values with different superscripts ($^{A\sim D}$) were significantly different between control and treatments by One-way ANOVA ($p<0.05$). (B) Ferritin was probed with anti-Ferritin antibody; lane 1: Control, lane 2: 1 μM SNP, lane 3: 10 μM SNP and lane 4: 100 μM SNP.

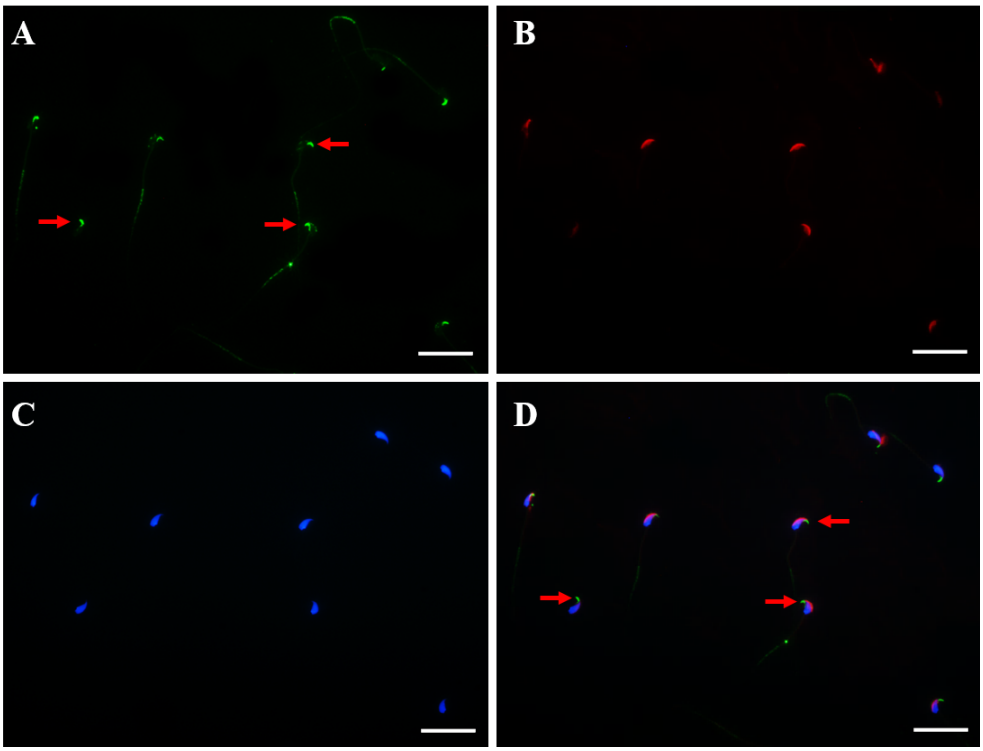


Fig. 2. Localization of Ferritin in spermatozoa. (A) Images of Ferritin (green) in sperm acrosome. (B) Images of the acrosome (lectin PNA, red) and tail (α-tubulin, red). (C) Nucleus (DAPI, blue). (D) Merged image of the nucleus, acrosome, tail, and Ferritin. Arrows directed Ferritin (A and D). Nikon TS-1000 microscope and NIS Elements image software (Nikon, Japan) were utilized to take the image. Bar=20 μm.

Effect of SNP on Spermatozoa Capacitation Status

To measure the effect of SNP on sperm capacitation status, we next performed chlortetracycline (CTC) staining. Increased numbers of acrosome reacted (AR pattern) spermatozoa were noted in response to treatment with all concentrations of SNP (p<0.05) (Fig. 3A), while levels of capacitated (B pattern) spermatozoa did not differ from control-treated spermatozoa (Fig. 3B). Moreover, SNP treatment resulted in a dose-dependent decrease in numbers of non-capacitated (F pattern) spermatozoa (p<0.05) (Fig. 3C).

Effect of SNP on Spermatozoa Phosphotyrosine Levels

To attain a mechanistic insight into SNP-induction of AR in spermatozoa, we next measured spermatozoa phosphotyrosine levels by western blot analysis normalized with α-tubulin (Fig. 4). We observed significant increases in ~17, ~40 and ~56 kDa tyrosine-phosphorylated proteins in SNP treated spermatozoa (p< 0.05) (Fig. 4).

Effect of SNP on Fertilization and Embryo Development

Next we used IVF to evaluate the effect of SNP on fertilization and embryo development. The rate of cleavage was significantly decreased in spermatozoa treated with higher concentrations (10 and 100 μM) of SNP (p<0.05) (Fig. 5A). Similarly, the rate of blastocyst formation was significantly decreased in a dose-dependent manner following treatment of the spermatozoa with

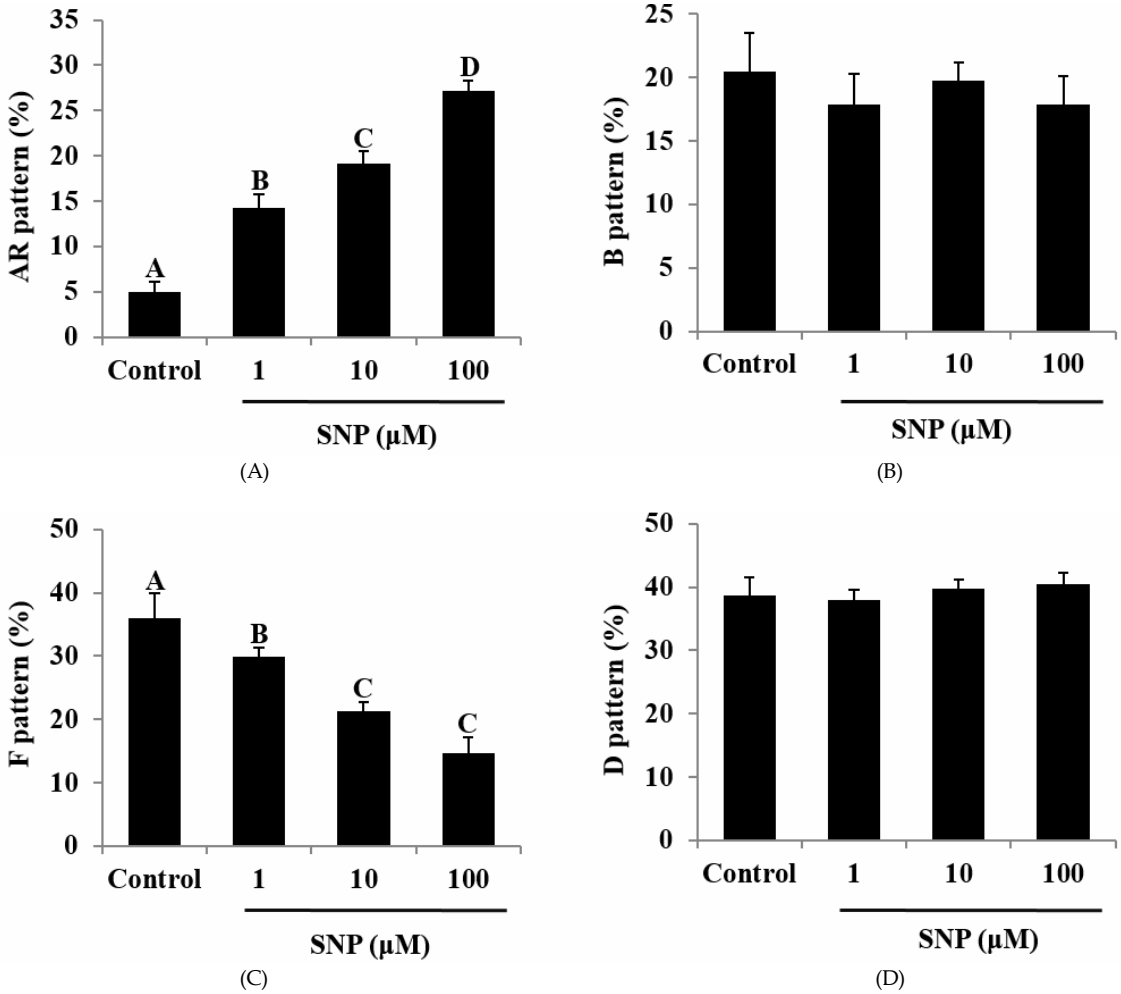
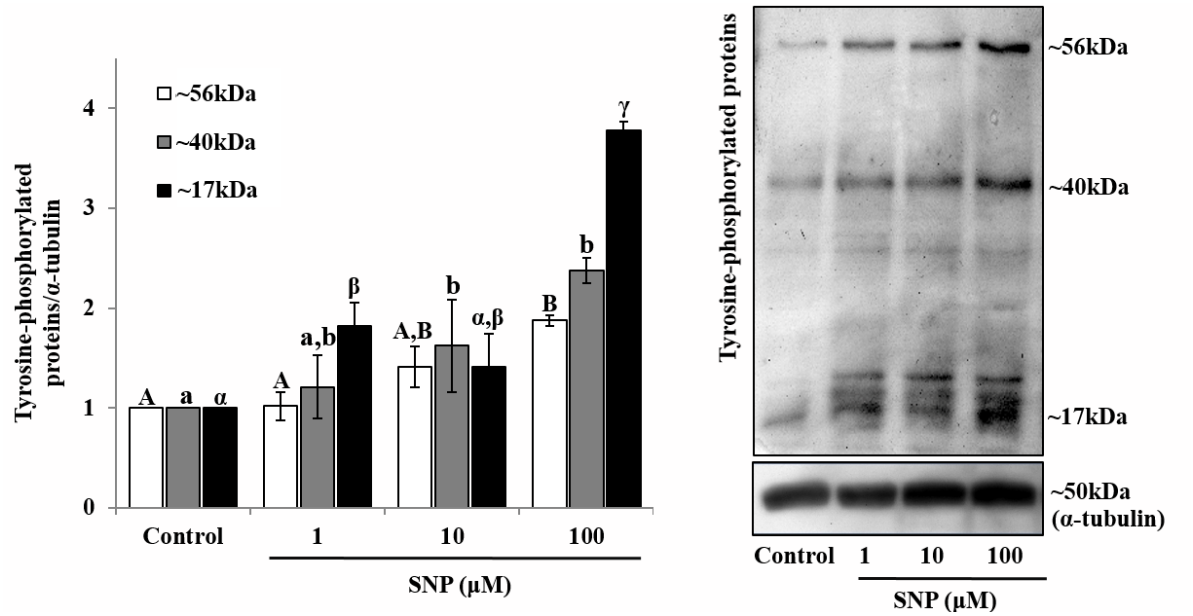
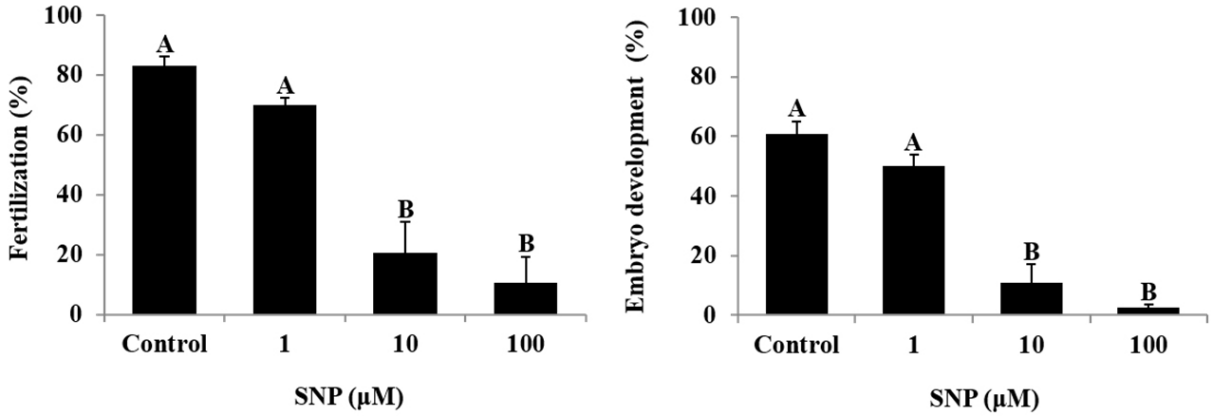


Fig. 3. Effect of SNP on the capacitation status of spermatozoa *in vitro*. (A) Appearance of acrosome reacted (AR pattern) spermatozoa in control and SNP treatment. (B) Changes in the number of capacitated (B pattern) spermatozoa in response to SNP. (C) Changes in the number of non-capacitated (F pattern) spermatozoa in response to SNP. (D) Changes in the number of dead (D pattern) spermatozoa in response to SNP. Data are the mean of 6 replicates±SEM. Values with different superscripts ($^{A\sim D}$) were significantly different between control and treatment groups by one-way ANOVA (p<0.05).



(A) (B)

Fig. 4. Effect of SNP on tyrosine phosphorylation in spermatozoa. (A) Density of tyrosine-phosphorylated proteins (black bar: ~56 kDa, grey bar: ~40 kDa and open bar: ~56 kDa) in response to control and various concentrations of SNP treatment. Data are the mean of 4 replicates±SEM. Values with different superscripts ($^{A,B,a,b,\alpha,\beta,\gamma}$) were significantly different between control and treatment groups by One-way ANOVA (p<0.05). **(B)** Tyrosine-phosphorylated proteins were probed with PY 20; lane 1: Control, lane 2: 1 μM SNP, lane 3: 10 μM SNP, lane 4: 100 μM SNP.



(A) (B)

Fig. 5. Effect of SNP on fertilization and embryonic development. (A) Fertility (%) in control sample and in response to SNP treatment. (B) Embryo development (%) under the same conditions. Data represent the mean of 3 replicate±SEM. Values with different superscripts (^{A,B}) were significantly different between control and treatment groups by One-way ANOVA (p<0.05).

SNP (p<0.05) (Fig. 5B).

DISCUSSION

Iron is an essential element in mammalian cells that is often toxic in excess, and a balance between iron accumulation, sequestration and release is obligatory to maintaining cell viability. While iron homeostasis in cells is known to be maintained by the highly-conserved Ferritin protein, little has been learned to date regarding the relationship between compromised iron homeostasis and Ferritin synthesis with sperm function. In this study, we provide mechanistic insights into the relationship between dysregulation of iron homeostasis and ma-

mmalian fertility.

We have demonstrated that SNP induces levels of Ferritin in spermatozoa (Fig. 1). In agreement with our previous findings, it is well appreciated that the SNP results in increases in cellular levels of iron and NO^+ (Kress *et al*., 2002) and that exposure of macrophages, fibroblast and hepatoma cells to SNP results in dramatic increase of Ferritin synthesis via the IRP-IREsystem (Kim and Ponka, 2002; Recalcati *et al*., 1998; Phillips *et al*., 1996). Based on these data and our own, we hypothesize that iron potentially stimulates the Ferritin synthesis in spermatozoa. Ferritin has been postulated to have a role in the sequestration of iron in a comparatively non-toxic form (Harrison and Arosio, 1996), and the increased Ferritin levels we observed might represent a detoxifying mechanism in response to the excess iron levels in spermatozoa treated with SNP. Ferritin levels in different cells are a function of a variety of factors, including the rate of transcription, the rate of mRNA translation, and the stability and storage of the translated mRNA. In support of our observations in spermatozoa, (Theil, 1987) and (Brenneisen *et al*., 1998) have hypothesized that free iron has direct effects on the production of Ferritin.

It has been reported that SNP increased tyrosine phosphorylation and the AR in a dose-dependent manner (Rahman *et al*., 2014). Our results also showed that the SNP-induced increase tyrosine phosphorylation was associated with a robust AR (Fig. 3 and 4). AR is important for successful fertilization and capacitation, and is associated with changes in sperm membrane fluidity, intracellular levels of ATP, cyclic adenosine monophosphate (cAMP) and ions (Ca^{2+}, Na^+, K^+, Cl^- and HCO_3^-), tyrosine phosphorylation and PKA activity (Baldi *et al*., 2000; Kwon *et al*., 2014a and b). To address the molecular mechanism underlying iron-induced AR, we examined the effect of iron on tyrosine phosphorylation. We found that increased tyrosine phosphorylation accompanied AR in spermatozoa (Fig. 3 and 4). Our results suggest therefore that iron-stimulated changes in ion transport across the sperm membrane are associated with AR.

It is also important to note that we confirmed the location of Ferritin in the acrosomal region of spermatozoa (Fig. 2). While the significance of this protein with respect to reproductive process is not known, it is tempting to speculate that the presence of Ferritin in the acrosome itself reflects a role for Ferritin in capacitation and the AR. Although our study provides no clear-cut evidence for this, further studies are underway to resolve this issue.

Finally, we evaluated the effect of iron on fertilization and embryonic development using an IVF system. Recently, Rahman *et al*. (2014) reported that SNP was decreased fertilization and embryo development. Our results also showed that the SNP was decreased fertilization and embryo development (Fig. 5). The present study suggests that this reduction in fertility is linked to a robust AR changes in spermatozoa. It has been demonstrated that spermatozoa from homozygous beta-thalassemia patients contain increased levels of iron (27～43 mM, normal range 9～30 mM) and Ferritin (996～4,385 μg/L, normal range 22～275 μg/L) (Perera *et al*., 2002). Although the SNP-induced increases in both parameters in the current study were not as dramatic, they were sufficient to profoundly compromise both fertilization and embryonic development. It has been reported that acute accumulation of iron in testis is implicated in oxidative damage to lipids, proteins and DNA in testicular cells (Slivkova *et al*., 2009). We speculate that this process adversely affects the ability of the spermatozoa to fertilize.

Successful fertilization requires that spermatozoa complete capacitation and AR. Premature AR, potentially resulting in altered mitochondrial function, can lead to a reduction of motility and failure of chromatin decondensation, which has consequences for spermatozoa viability and fertility (Chaveiro *et al*., 2006, Wongtawan *et al*., 2006; *et al*., 2005). Under normal circumstances, a component of the zona pellucida (ZP) of the unfertilized female egg induces sperm to undergo AR following binding. In contrast, spermatozoa in which capacitation and AR have taken place prematurely, prior to the spermatozoa reaching the ZP, are unable to penetrate the ZP, with consequent loss in fertilizing capacity (Florman and Ducibella, 2006).

Collectively, our findings support the notion that SNP-induced alterations in sperm iron and Ferritin levels modulate cellular tyrosine phosphorylation ultimately resulting in a robust AR. Moreover we suggest that compromised fertilization and embryonic development is related to the altered AR. In summary, iron overload has a profound effect on critical sperm functions, fertilization, embryonic development, and has considerable implications for male fertility. We anticipate that extraneous iron sources might be profitably pursued as targets for future development of male contraceptives.

REFERENCES

1. Baldi E, Luconi M, Bonaccorsi L, Muratori M, Forti G (2000): Intracellular events and signaling pathways involved in sperm acquisition of fertilizing capacity and acrosome reaction. Front Biosci 1:E110-E123.

2. Borkowska A, Sielicka-Dudzin A, Herman-Antosiewicz A, Halon M, Wozniak M, Antosiewicz J (2011): P66Shc mediated ferritin degradation–a novel mechanism of ROS formation. Free Radic Biol Med 51:658-663.
3. Brenneisen P, Wenk J, Klotz LO, Wlaschek M, Briviba K, Krieg T, Sies H, Scharffetter-Kochanek K (1998): Central role of ferrous/ferric iron in the ultraviolet B irradiation-mediated signaling pathway leading to increased interstitial collagenase (matrix-degrading metalloprotease (MMP)-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts. J Biol Chem 273:5279-5287.
4. Chaveiro A, Machado L, Frijters A, Engel B, Woelders H (2006): Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic supports. Theriogenology 65:1875-1890.
5. Comporti M (2002): Introduction-serial review: iron and cellular redox status. Free Radic Biol Med 32: 565-567.
6. Cotter PZ, Goolsby HA, Prien SD (2005): Preliminary evaluation of a unique freezing technology for bovine spermatozoa cryopreservation. Reprod Domest Anim 40:98-99.
7. Florman HM, Ducibella T (2006): Fertilization in Mammals. Knobil and Neill's Physiology of Reproduction (Elsevier, Academic Press, St. Louis, MO) Vol 1, 3rd ed, pp 55-112.
8. Harrison PM, Arosio P (1996): The ferritins: molecular properties, iron storage function and cellular regulation. Biochim Biophys Acta 1275:161-203.
9. Kamboj VP, Kar AB (1964): Effect of iron salts on the genital organs and fertility of male rats. Acta Biol Med Ger 13:928-945.
10. Kim HJ, Tsoy I, Park MK, Lee YS, Lee JH, Seo HG, Chang KC (2006): Iron released by sodium nitroprusside contributes to heme oxygenase-1 induction via the cAMP-protein kinase A-mitogen-activated protein kinase pathway in RAW 264.7 cells. Mol Pharmacol 69:1633-1640.
11. Kim J, Kwon WS, Rahman MS, Lee JS, Yoon SJ, Park YJ, You YA, Pang MG (2015): Effect of sodium fluoride on male mouse fertility. Andrology 3:544-551.
12. Kim S, Ponka P (2002): Nitrogen monoxide-mediated control of ferritin synthesis: implications for macrophage iron homeostasis. Proc Natl Acad Sci USA 99:12214-12219.
13. Kress GJ, Dineley KE, Reynolds IJ (2002): The relationship between intracellular free iron and cell injury in cultured neurons, astrocytes, and oligodendrocytes. J Neurosci 22:5848-5855.
14. Kwon WS, Park YJ, Kim YH, You YA, Kim IC, Pang MG (2013a): Vasopressin effectively suppresses male fertility. PLoS ONE 8:e5419.
15. Kwon WS, Park YJ, Mohamed el-SA, Pang MG (2013b): Voltage-dependent anion channels are a key factor of male fertility. Fertil Steril 99:354-361.
16. Kwon WS, Rahman MS, Lee JS, Kim J, Yoon SJ, Park YJ, You YA, Hwang S, Pang MG (2014a): A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics 15:897.
17. Kwon WS, Rahman MS, Lee JS, You YA, Pang MG (2015): Improving litter size by boar spermatozoa: application of combined H33258/CTC staining in field trial with artificial insemination. Andrology 3: 552-557.
18. Kwon WS, Rahman MS, Pang MG (2014b): Diagnosis and prognosis of male infertility in mammal: the focusing of tyrosine phosphorylation and phosphotyrosine proteins. J Proteome Res 13:4505-4517.
19. Lee JS, Kwon WS, Rahman MS, Yoon SJ, Park YJ, Pang MG (2015): Actin-related protein 2/3 complex-based actin polymerization is critical for male fertility. Andrology 3:937-946.
20. Lucesoli F, Caligiuri M, Roberti MF, Perazzo JC, Fraga CG (1999): Dose-dependent increase of oxidative damage in the testes of rats subjected to acute iron overload. Arch Biochem Biophys 372:37-43.
21. Maxwell WM, Johnson LA (1999): Physiology of spermatozoa at high dilution rates: the influence of seminal plasma. Theriogenology 52:1353-1362.
22. Perera D, Pizzey A, Campbell A, Katz M, Porter J, Petrou M, Irvine DS, Chatterjee R (2002): Sperm DNA damage in potentially fertile homozygous beta-thalassaemia patients with iron overload. Hum Reprod 17:1820-1825.
23. Phillips JD, Kinikini DV, Yu Y, Guo B, Leibold EA (1996): Differential regulation of IRP1 and IRP2 by nitric oxide in rat hepatoma cells. Blood 87:2983-2992.
24. Puntarulo S (2005): Iron, oxidative stress and human health. Mol Aspects Med 26:299-312.
25. Rahman MS, Kwon WS, Lee JS, Kim J, Yoon SJ, Park YJ, You YA, Hwang S, Pang MG (2014): Sodium nitroprusside suppresses male fertility *in vitro*. Andrology 2:899-909.
26. Rahman MS, Kwon WS, Lee JS, Yoon SJ, Ryu BY, Pang MG (2015): Bisphenol-A affects male fertility via fertility-related proteins in spermatozoa. Sci Rep 5:9169.
27. Rajesh Kumar T, Doreswamy K, Shrilatha B, Muralidhara (2002) Oxidative stress associated DNA damage in testis of mice: induction of abnormal sperms and effects on fertility. Mutat Res 513(1-2): 103-111.

28. Recalcati S, Taramelli D, Conte D, Cairo G (1998): Nitric oxide-mediated induction of ferritin synthesis in J774 macrophages by inflammatory cytokines: role of selective iron regulatory protein-2 downregulation. Blood 91:1059-1066.
29. Slivkova J, Popelkova M, Massanyi P, Toporcerova S, Stawarz R, Formicki G, Lukac N, Putała A, Guzik M (2009): Concentration of trace elements in human semen and relation to spermatozoa quality. J Environ Sci Health A Tox Hazard Subst Environ Eng 44:370-375.
30. Theil EC (1987): Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. Annu Rev Biochem 56:289-315.
31. Wellejus A, Poulsen HE, Loft S (2000): Iron-induced oxidative DNA damage in rat sperm cells *in vivo* and *in vitro.* Free Radic Res 32:75-83.
32. Wise T, Lunstra DD, Rohrer GA, Ford JJ (2003): Relationships of testicular iron and ferritin concentrations with testicular weight and sperm production in boars. J Anim Sci 81:503-511.
33. Wongtawan T, Saravia F, Wallgren M, Caballero I, Rodríguez-Martínez H (2006): Fertility after deep intra-uterine artificial insemination of concentrated low-volume boar semen doses. Theriogenology 65: 773-787.

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