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Original Contribution

Effects of sperm DNA damage on the levels of RAD51 and p53 proteins in zygotes and 2-cell embryos sired by golden hamsters without the major accessory sex glands

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ABSTRACT

We previously reported that the male accessory sex gland (ASG) secretion is the main source of antioxidants to safeguard sperm genomic integrity and functional competence. Removal of all ASGs in the golden hamster can reduce male fertility by increasing embryo wastage. This study aims to investigate whether the oxidative DNA-damaged sperm from hamsters without all ASGs (TX) could successfully fertilize oocytes and to qualify the status of DNA repair by the expression of RAD51 and p53 proteins. Here we demonstrated a significantly higher DNA-base adduct formation (8-hydroxy-2′-deoxyguanosine) in sperm from TX males than those from sham-operated males. Comet assays demonstrated that all female pronuclei in both zygotes were intact, but single- and double-strand DNA damage was found in decondensed sperm in TX males only. DNA damage could also be detected in both nuclei of the TX 2-cell embryos. RAD51, a DNA repair enzyme, was found to be evenly distributed in the cytoplasm and nuclei in oocytes/zygotes, while at the 2-cell stage, a strong expression of p53 protein and a larger clear perinuclear area without RAD51 expression were found in TX embryos. In conclusion, we demonstrated for the first time DNA damage in decondensed sperm of zygotes and blastomeres of 2-cell stage embryos sired by TX males, resulting in the activation of DNA repair. Sperm DNA damage could induce the increase in p53 expression and the reduction of RAD51 expression in the TX 2-cell stage embryos.

Introduction

Reactive oxygen species (ROS) are free radicals such as superoxide anions ($O^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). An optimal amount of ROS in sperm favors capacitation, acrosome reaction, and fertilization processes [18,29]. In the post-coital female reproductive tract, the amount of ROS depends on their production by sperm and leucocytes and their sequestration by antioxidants in the secretions of both male and female. If the balance is upset, spermatozoa may experience oxidative stress such that structural integrity and/or functional competence may be impaired [35,38] and finally male factor infertility occurs [12]. The sperm plasma membrane is particularly vulnerable to oxidative stress because of the presence of NADPH oxidase and a high content of polyunsaturated fatty acids (PUFA) that give fluidity for membrane changes during maturation and fertilization [5]. Accessory sex glands (ASGs) and their secretions play an important role in male reproductive function by forming the seminal plasma of the ejaculate. In human, the seminal plasma contains a variety of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and free radical scavengers such as vitamins C and E, hypotaurine, taurine, uric acid, and albumin [24,40,44]. In the golden hamster, activities of SOD, CAT and GPx have been demonstrated in the major male accessory sex glands secretions [11]. Of these, SOD is the predominant one and it has been found to play a significant role in protecting sperm against oxidative stress induced by NADPH treatment in vitro [11].

Abbreviations: ASG, male accessory sex gland; BN, blastomere nuclei; 53BP1, p53-binding protein 1; BSA, bovine serum albumin; CAT, catalase; dsDNA, double strand DNA; FN, female pronucleus; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; HR, homologous recombination; $O^{\cdot-}$, superoxide anions; $OH^{\cdot-}$, hydroxyl radicals; 8OHdG, 8-hydroxy-2′-deoxyguanosine; PB, polar body; PBS, phosphate-buffered saline; *p.c.*, post coital; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species; RT, room temperature; SH, sham-operated male hamster; Sp, sperm nucleus; ssDNA, single strand DNA; TALP, Tyrode's albumin lactate pyruvate medium; TX, all ASGs removed male hamster

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We have demonstrated using single-cell gel electrophoresis (or comet assay) that the sperm produced by male hamsters with major accessory sex glands ablated had a higher level of single- and double-strand DNA damage [10]. We have also shown that embryos derived from fertilization with such sperm have more structural abnormality and lethality, as well as a slower cell division [9,14,23,30,39].

Oxidative DNA damage in sperm has been demonstrated by direct staining of sperm with 8-hydroxy-2′deoxyguanosine (8OHdG) and the result correlated with DNA fragmentation [4]. Spermatozoa cannot repair damaged DNA on their own because they lack a cytoplasmic enzyme system [3,6]. On the other hand, oocytes and preimplantation embryos have a high level of DNA repair enzyme activity. The capacity to repair DNA damage induced by UV irradiation or alkylating agents by pre- and postreplication repair mechanisms has been demonstrated from the time the sperm enters the egg to the first cleavage metaphase [19,22,27,28]. The DNA repair enzyme, RAD51, is considered to play a pivotal role in homologous recombination (HR) because it is able to repair damaged dsDNA. The tumor suppressor factor p53 is implicated in the regulation of cell growth and DNA repair by interacting with RAD51 [7,25].

Therefore, the aim of this study is to investigate whether the oxidative DNA-damaged sperm produced by hamsters without the major paternal accessory sex glands could successfully fertilize oocytes. The expressions of RAD51 and p53 proteins in zygotes and 2-cell embryos were also studied to infer the status of DNA repair.

Materials and methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. All chemicals used in comet assays were described in previous reports [10].

Animal model

Animals were maintained and handled in compliance with a protocol approved by the Committee on the Use of Life Animals for Teaching and Research of The University of Hong Kong. Randomly bred golden hamsters (*Mesocricetus auratus*) were supplied by and housed in the Laboratory Animal Unit, Faculty of Medicine of The University of Hong Kong. They were kept at 22 °C under 14 h light–10 h dark lighting cycle (with lights on from 11:00 am to 01:00 am). Food and tap water were available ad libitum. Vaginal smears of 6- to 8-week-old female hamsters were checked daily for at least two normal consecutive cycles before mating. Under general anaesthesia with a 2:1 mixture of 10% ketamine and 2% xylazine (0.2 mL/100 g of body weight, Alfasan, Woerden, Holland) given intraperitoneally, all major male accessory sex glands (the ampullary glands, ventral prostate, dorsolateral prostates, coagulating glands, and seminal vesicles) were surgically ablated according to procedures described by Chow et al. [15] in the TX group ($n=10$); the control hamsters were sham-operated (SH group; $n=8$). All animals were allowed to recuperate for 1 month and the success of surgery was confirmed postmortem. The following experiments were all taken blindly, including detection of oxidative DNA damage of sperm, assessment of DNA damage of zygote/embryo, and localization of RAD51 and p53.

Sample preparation

Collection of sperm and 8-hydroxy-2′-deoxyguanosine staining

The two groups of male hamsters, TX and SH, were mated individually with a female hamster on the day of estrus for 15 min. The female was sacrificed with an overdose of sodium pentobarbital injected intraperitoneally and sperm was flushed out from the uterus with 0.01 M PBS. The sperm was treated with 3% H_2O_2 and incubated for 10 min and then smeared on a silianized slide and air-dried. The slide was fixed in cold 100% methanol (-20 °C) and kept at -20 °C until use. Nonspecific binding sites were blocked with 1% BSA in 0.01 M PBS for 30 min. The negative controls were performed without primary antibodies or with isotype of mouse IgG. For 8OHdG, the slides were incubated with 1:50 primary antibody (mouse anti-8-oxo-guanine, Trevigen Inc., Gaitnersburg, MD) at 4 °C overnight. The slides were washed thrice with 0.01 M PBS and incubated with goat anti-mouse FITC for 30 min at room temperature (RT) in a dark and humid chamber. After rinsing, the slides were mounted with an anti-fade VECTASHIELD Mounting Medium (VECTOR, Burlingame, CA). Immunostaining signals were examined under an epifluorescent microscope equipped with an exciter filter (BP 450–490) and a barrier filter (LP520). At least 100 sperm were counted from each animal and the percentage of positively stained sperm was recorded.

Collection of zygotes and 2-cell embryos for comet assay

Each normally cycling female hamster was mated with one operated male for 15 min on the day of estrus. They were sacrificed with an overdose of sodium pentobarbital injected intraperitoneally 5 h or 22 h later to collect zygotes and 2-cell embryos respectively. Zygotes and 2-cell embryos were collected from the oviducts by flushing with TALP-BSA medium. Cumulus cells were removed by incubating with 0.1% (w/v) hyaluronidase for a few minutes. The zona-intact zygotes and 2-cell embryos were washed three times in fresh TALP medium, transferred to 1X PBS/PVP (4 mg/mL), and assessed for ssDNA and dsDNA damage by alkaline and neutral comet assays, respectively.

Collection of unfertilized oocytes, zygotes, and 2-cell embryos for colocalization of RAD51 and p53 protein

Unfertilized oocytes and zygotes at 8 h p.c. and 2-cell embryos at 24 h p.c. were collected in 0.01 M PBS containing 4 mg/mL PVP and prepared as described in the last section. The zona was removed by incubation in acid Tyrode's medium for 1–2 min, followed by recovery in fresh medium containing 1% BSA and 4 mg/mL PVP for 15 min at 37 °C. Subsequently, these zona-free unfertilized oocytes, zygotes, and 2-cell embryos were embedded in 60 μL 0.5% (w/v) agarose 3:1 (Ameresco, Solon, OH) precoated on prewarmed CometSlides (Trevigen, Gaithersburg, MD) on a warm plate (37 °C) for colocalization of RAD51 and p53 protein.

Assessment of DNA damage in zygotes/embryos by comet assay

The comet assay adapted from Shen and Ong [34] was performed in the dark. Prewarmed CometSlides were coated with 50 μL 0.5% (w/v) agarose 3:1 on a warm plate at 50 °C. Zygotes were submersed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% (v/v) DMSO, and 1% (v/v) Triton X-100, pH 10) for 22 h 4 °C. Two-cell embryos were submersed in a modified cold lysis buffer (same as that used for zygote samples but with the addition of 1% *N*-lauryl sarcosine sodium [Ameresco] and 2% β-mercaptoethanol) for 2 h at 4 °C in the dark.

In the alkaline comet assay, the slides containing the zygotes/embryos were transferred to an electrophoresis chamber (Hoefer Pharmacia Biotech) filled with alkaline buffer (300 mM NaOH [E. Merck, Darmstadt, Germany], 1 mM EDTA, 0.2% (v/v) DMSO, and 0.1% (w/v) 8-hydroxyquinoline, pH > 13) and allowed 20 min for unwinding the DNA. The samples were then electrophoresed at 0.83 V/cm, 300 mA for 10 min at RT. Upon completion of electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH 7.4)