Hydroxytyrosol effectively improves the quality of pig sperm at room temperature

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Research

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Abstract

Background: Artificial insemination technology is the production technology used in more than 99% of the world's pig farms. Preservation of semen is mainly conducted at room temperature (17°C), during which the accumulation of reactive oxygen species (ROS). In our research, by adding different concentrations of hydroxytyrosol to the diluent during the storage of boar semen at 17°C, we compared them with the traditional diluent to study the cumulative amount of ROS, the extent of membrane damage, the sperm acrosome integrity, the activity of antioxidant enzymes, and other indicators to evaluate the effect of hydroxytyrosol on the changes of active oxygen and sperm quality during storage. After we determined the optimal amount of hydroxytyrosol in the diluent, Proteomics was used to monitor difference in sperm protein expression between the control group and the addition of 120 μmol/L hydroxytyrosol (optimum concentration) group after three days' storage. Ultimately, we selected the control group and the addition of 120 μmol/L hydroxytyrosol group to test the effect of artificial insemination.

Results: During storage of pig semen at room temperature, the sperm quality of the hydroxytyrosol-treated group was significantly improved ($P < 0.05$). Besides, its antioxidant capacity was also significantly improved ($P < 0.05$). Proteomics sequencing analysis proved that the addition of hydroxytyrosol treatment group has potential value in improving sperm cell quality. The significant increase in sow pregnancy rate and the increase in piglet birth weight also confirmed that sperm quality during storage can be of vital importance in actual production ($P < 0.05$).

Conclusions: Based on our results, we demonstrated that the addition of hydroxytyrosol to the diluent can not only improve the quality of pig sperm, but also has an irreplaceable role in improving the efficiency of artificial insemination.

Backgrounds

Pig artificial insemination (AI) has been widely used in recent years, and the sperm storage at room temperature has important significance in commercial production [7, 18]. Boar sperm are extremely sensitive to changes in temperature and are prone to cold shock [12, 28], Cold shock severely affects sperm quality and reduces the fertilization potential of sperm [22, 27]. Besides, high level of technology and equipment are required for cryopreservation of pig sperm, so researching the preservation of semen at room temperature has an irreplaceable role [11, 25]. However, during the semen preservation, sperm suffers from severe oxidative stress due to the accumulation of ROS which in turn decrease the sperm quality, compromise to the plasma membrane, the acrosome, the DNA integrity and the protein [10, 20, 24]. Besides, sperm with too high DNA fragment may even bring genetic disease to the offspring through sperm [16]. The key to solving the problem of ROS accumulation during pig semen preservation is the formulation of the diluent [6].

In recent years, natural antioxidants are being largely investigated owing to their outstanding oxidation resistance [5]. Hydroxytyrosol is a natural active oxygen scavenger known as ROS scavenger [19]. Besides, many studies have reported that hydroxytyrosol can effectively solve the problem of accumulation of ROS in various cells. For instance, hydroxytyrosol can protect vascular endothelial cells from the cytotoxic effects of hydrogen peroxide [29]. Studies have shown that hydroxytyrosol reduces the level of active oxygen accumulation and DNA fragmentation in human sperm [13]. To the present researches, few studies have shown that the addition of hydroxytyrosol to diluents have a positive effect in storing semen at room temperature. Due to the great antioxidant effect of the hydroxytyrosol, it was hypothesized that the hydroxytyrosol to sperm diluent might improve sperm quality and reduce sperm damage caused by oxidative stress.

The aim of the present study was to assess the influence of the addition of different concentrations of hydroxytyrosol to sperm extender. We have demonstrated that hydroxytyrosol is effective for the preservation of boar semen stored at room temperature and the use of hydroxytyrosol is a suitable alternative for the traditional dilution. The objective of our study was to investigate whether adding hydroxytyrosol to sperm diluent could improve sperm quality and fertility of liquid pig semen stored at room temperature.

Materials And Methods

Chemicals and sources

Unless otherwise stated, all chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The extender used in this study was Beltsville thawing solution (BTS), the diluent was composed of 37.5g fructose, 1.25 g of ethylenediaminetetraacetic acid (EDTA)-Na2, 6.0g sodium citrate, 6.0 g of citric acid two sodium, 0.75g of potassium chloride, 1.25g of $NaHCO_3$ and 0.2 mg of gentamicin in 1000mL of deionized water. The only difference between control group and treatment groups is the addition of hydroxytyrosol. Samples of control and treatment groups repeated at least five times.

Semen collection

The experimental animals were selected 6 healthy Large White boars from the national core conservation farms in Xingping City, Shaanxi Province (34° 12’ N, 108° 17’ E). The boars are in the same housing and management conditions, the environment is controlled at 15-25°C, individual fences in the building, the windows are exposed to natural daylight and supplemental light, a total of 16 hours of light per day (at the boar eye level ≥ 150lx light intensity). According to the nutritional needs of adult AI boars, they can get water freely and feed commercial forage. The test was carried out by hand-collecting, and the semen was collected and filtered twice with 0.22 μm filter membrane. Besides, the first pre-sperm fraction was not collected and the gelatinous portion was discarded. The sperm motility was detected by computer-assisted sperm analysis CASA system (Hamilton Thorne Research, Beverly, MA, 87 USA). The test used only semen samples with ejaculation volume ≥200 ml, milky white, and slightly smelly, with vitality >85%.

Use BTS (Beltsville Thawing Solution) to dilute semen to adjust the concentration to approximately 1.5×10^7 sperm/ml. Various concentrations of hydroxytyrosol (0, 40, 80, 120, 160, 200 μM) were added to the sperm dilution.

Sperm motility assay

The sperm motility of each preservation group was tested using the CASA system and was measured at 0, 1, 2, 3, 4, 5 days. The method is as follows: 8 μL of each group is pre-warmed on a clean slide to be observed at 37°C. The CASA system automatically detects the sperm motility and related motion parameters in the field of view (A minimum of 300 sperm cells were recorded for each sample from five randomly selected fields per replicate).

Analysis of ROS content

The ROS accumulation was measured at 0, 1, 3, 5 days during liquid preservation of semen. 300 μL of semen was taken from each semen sample, 10 μL of the active oxygen fluorescent probe DCFH-DA was added, and incubation was carried out at 37°C for 30 minutes. Flow cytometry was used to detect the fluorescence intensity of the probe (*Nanjing Institute of Bioengineering, Co., Nanjing, China (Product No.: E004-1-1*)).

Analysis of plasma membrane and acrosome integrity

The sperm membrane and acrosome integrity were measured at 0, 1, 3, 5 days during liquid preservation of semen [8]. Sperm membrane: 0.1 μL SYBR-14 working solution, 0.5 μL PI (propidium iodide) working solution and 80 μL pig sperm used water bath heating 10 minutes at 37°C. 10 μL was taken on a clean glass slide and the slide was observed at 37°C . Acrosome staining: 0.1 μL DAPI (4', 6-diamidino-2-phenylindole) working solution was pre-incubated with 80 μL sperm for 30 minutes at 37°C, then 25 μL of uniform smear was taken. After fixation for 10 minutes in methanol, 5 μL of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) working droplets were stained, after incubating for 20 minutes at 37°C, the slides were taken and observed. Each field of view was not less than 300 sperm. The sperm plasma membrane and the acrosome integrity were detected using an inverted fluorescence microscope (Leica DMI8). The regression equation was obtained by least squares analysis. Image Pro-Plus software was used to quantify fluorescence intensity.

Pig sperm DNA damage test (comet electrophoresis test)

When the pig semen was preserved to day 3, the DNA damage of the control group and the hydroxytyrosol 120 μmol/L group were observed by the comet assay. The test method was carried out in strict accordance with the instructions of the DNA Damage Detection Kit (*Nanjing Institute of Bioengineering*, Co., *Nanjing, China (Product No.: G010 -1-1)*).

Analysis of semen antioxidant ability

The MDA competencies were assessed using a malondialdehyde test kit (*Nanjing Institute of Bioengineering*, Co., *Nanjing, China (Product No.: A007-1-1*)) according to the manufacturer's instructions. Briefly, the samples and test reagent were mixed by vortexing. The tubes were then sealed with plastic wrap and punctured with a small needle to produce a small hole. The samples were used water bath heating 40 minutes at 95°C. When the water cooling, centrifuged 10 minutes at 1500g. The supernatants were collected and the absorbance was measured at 532 nm in the fluorescent microplate reader (*Boster, Co., USA*).

The CAT and T-AOC were determined using kits according to the manufacturer's protocols (*Nanjing Institute of Bioengineering*, Co., *Nanjing, China* (*Product No.: A007-1-1, A015-1-2*)). The preparation of the sample followed the instructions of the operation. Finally, the CAT activity was measured at 405 nm and T-AOC was measured at 520 nm in the fluorescent microplate reader.

SOD and GSH-PX in this study were performed by SOD kit and GSH-PX kit according to the manufacturer's protocols (*Nanjing Institute of Bioengineering*, Co., *Nanjing, China* (*Product No.: A001-3-2, A005-1-2*). The absorbance of samples was determined at 550 nm for SOD and 412 nm for GSH-PX at the end of reaction in the fluorescent microplate reader.

Sequencing analysis of pig sperm protein group

Protein sample was sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 12,000 g at 4°C for 10 minutes. Ultimately, the supernatant was collected and the protein concentration was determined with BCA kit according to the manufacturer's instructions.

Trypsin Digestion:

For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration less than 2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratios for a second 4 h-digestion.

TMT Labeling (optional):

After trypsin digestion, peptide was desalted by Strata XC18SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for TMT kit/iTRAQ kit. Briefly, one unit of TMT/iTRAQ reagent was thawed and reconstituted in acetonitrile. The peptide mixtures were then incubated for 2 hours at room temperature and pooled, desalted and dried by vacuum centrifugation.

HPLC Fractionation (optional):

The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, and 250 mm length). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into 18 fractions and dried by vacuum centrifuging.

LC-MS/MS Analysis:

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15-cm length, 75 μm i.d.). The gradient was comprised of an increase from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive^{TM}Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z.

Database Search:

The resulting MS/MS data were processed using the Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against databases concatenated with the reverse decoy database. Trypsin/P was specified as a cleavage enzyme allowing up to 2 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in the First search and 5 ppm in the Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and oxidation on Met was specified as variable modifications. FDR was adjusted to < 1% and the minimum score for peptides were set > 40.

Artificial insemination test

The experimental animals were selected from the Huayang breeding farm in Luonan County, Shaanxi Province (33° 52’ N, 109° 44’ E), with 198 healthy Large White sows and a small difference in body weight.

Pre-treatment of each concentration of pig semen by artificial insemination, batching the test sows in batches (33 batches per batch, preferentially selecting sows with good estrus conditions and recent uninfected diseases, completing all breeding within 10 days), Maintain the same nutritional physiology and management level during breeding. The sow was fertilized in the morning and fertilized again the

next morning. Artificial insemination is operated by skilled technicians in accordance with standard artificial insemination procedures. Each sow is inseminated with 40 to 50 ml of semen.

After 35 days of artificial insemination, the hand-held ultrasonic detector was used to check the pregnancy status of the sow. When the sows were born, the size of litters were checked and recorded, and the newborn weight of the piglets were also weighed.

Statistical analysis

All results were expressed as the mean ± SD. Sperm activity, plasma membrane integrity, acrosome integrity, T-AOC activity, MDA content, CAT activity, ROS accumulation, SOD content and GSH-PX activity were compared using Duncan's multiple-range test. Statistical analyses were performed using Statistical Product and Service Solutions (SPSS 21; SPSS, Chicago, IL, USA). Statistically significant differences between variable were determined at $P < 0.05$.

Results

Hydroxytyrosol improves the preservation of pig semen at 17°C

After adding 40, 80, 120, 160, 200 μmol/L hydroxytyrosol to the diluent formulation, sperm motility was higher than the control group from day 2 (Figure1.a). From day 3, the mean fluorescence intensity (MFI) of different concentrations of hydroxytyrosol's DCF were significantly lower than the control group (Table1, $P < 0.05$), while the MF of the 120 μmol/L hydroxytyrosol addition group was significantly lower than the other groups ($P < 0.05$). When the pig semen was preserved until day 5, the ROS of each group increased rapidly, and the overall peak of the DCF fluorescence peak shifted to the right (Figure1.b). The Average fluorescence intensity doubled from day 3, and the average fluorescence intensity of the control groups (MFI) reached 7047, which was significantly higher than the hydroxytyrosol groups ($P < 0.01$). Besides, the 120 μmol/L hydroxytyrosol group was maintained at a relatively low level compared with other groups (Table 1, $P < 0.05$), which indicated that the addition of 120 μmol/L significantly reduced the accumulation of ROS during semen preservation.

During semen preservation, the plasma membrane integrity rate of each test group decreased with the prolonging of preservation time, which may be due to membrane lipid peroxidation caused by ROS accumulation [4, 21]. After the addition of hydroxytyrosol to the diluent, the plasma membrane damage rate of the sperm was significantly decreased ($P < 0.05$), especially the sperm membrane with the 120 μmol/L hydroxytyrosol addition group was the best. Besides, when the semen was preserved to day 5 (Figure2.a), this group showed significant difference from other groups ($P < 0.05$).

The acrosome integrity of each group decreased with longer semen preservation time. However, there was no significant difference between the groups ($P > 0.05$). On the third day of preservation, although the acrosome integrity of the groups added with hydroxytyrosol were higher than the control group, the statistical difference was still not significant ($P > 0.05$). When there were large differences in sperm

motility and plasma membrane, the group treated with 120 μmol/L hydroxytyrosol did not show a clear advantage on the third day (Figure2.c).

By observing the DNA damage of the sperm added to the 120 μmol/L hydroxytyrosol group and the control group on the third day of semen preservation, we found that the fragmentation was significantly inhibited (Figure2.e).

Hydroxytyrosol slows the rate of decline in sperm antioxidant enzyme activity

During the whole course of semen preservation, the total antioxidant capacity of pig sperm gradually decreased with time passing by (Figure3.a). When stored on the day 1, the total antioxidant capacity of the hydroxytyrosol groups were significantly higher than that of the control group ($P < 0.05$). On the day 3, although there were no significant differences within the hydroxytyrosol added groups ($P > 0.05$), there were still two groups (HT 120 μmol/L, 200 μmol/L) were significantly different from the control group. Besides, the addition of 120 μmol/L hydroxytyrosol continued to perform well in the 5 days of preservation, significantly superior to the other groups in total antioxidant capacity ($P < 0.05$). It indicated that the preservation method of hydroxytyrosol treatment increased the total antioxidant capacity of pig semen in a dose-dependent manner.

Glutathione peroxidase activity decreased gradually with the prolonged semen preservation (Figure3.b). On the day 3, the 120 μmol/L hydroxytyrosol treatment group showed significant difference from other groups ($P < 0.05$). When stored on the day 5, although the glutathione peroxidase activity of each group decreased sharply, the enzyme activity in the hydroxytyrosol groups was higher than in the control group. Especially the hydroxytyrosol 120 μmol/L group mentioned above, which was significantly higher than the other groups ($P < 0.05$).

From day 1, the SOD of the control group was at a lower level, while the hydroxytyrosol group showed a higher SOD level (Figure3.c). From day 1, the 120 μmol/L hydroxytyrosol group showed a high value and was significantly different compared with other groups ($P < 0.05$). Our result showed that treatment group added hydroxytyrosol could effectively improve the activity of SOD during preservation.

The day 1 result showed the catalase activity of the hydroxytyrosol treatment group was significantly higher than the control group (Figure3.d) ($P < 0.05$). Besides, treatment group was significantly higher than the day 0, indicating that sperm H_2O_2 was also accumulating. The increase in systemic enzyme activity is a sign showing that sperm responds to this condition. On the third day of preservation, the catalase activity reached its peak, and the hydroxytyrosol test group showed significant difference compared with the control group ($P < 0.05$). On the fifth day of preservation, the catalase activity of each group decreased significantly (Figure3.d) ($P < 0.05$).

During the whole semen preservation, the malondialdehyde (MDA) content of pig sperm increased during the storage time (Figure3.e). On day 1, the malondialdehyde content of the control group was higher than other groups. When saving to day 3, the malondialdehyde content of the test groups were significantly

lower than that of the control group ($P < 0.05$). Our result indicated that the membranous peroxidation of the control group is more serious, and consistent with the integrity of the plasma membrane in previous tests. When the pig semen was preserved on day 5, the malondialdehyde content increased sharply, and the pig sperm membranous system was severely damaged. However, the content of malondialdehyde in the hydroxytyrosol treatment groups were lower than in the control group and the malondialdehyde content in the treatment group with the added concentration of HT 120 μmol/L was the lowest ($P < 0.05$).

Proteomics difference between hydroxytyrosol treatment group and control group was significant

In this experiment, a total of 259,816 secondary spectrums were obtained by mass spectrometry (Figure4. a). After the mass spectrum, secondary spectrum was searched by protein theory data, 33704 effective spectrums were obtained, with a 13.0% rate of spectrum utilization, the available effective spectrum number was 33704, and the spectrum utilization rate was 13.0%. The spectral analysis identified a total of 17,615 peptides, with a specific peptide of 16513. Our results showed that among the total of 2832 proteins, 2483 proteins were quantifiable (quantitative proteins indicate at least one comparative group with quantitative information).

The threshold of change was expressed by 1.3 times (Fold Change=1.3), and the t-test $P < 0.05$ was used as the significant threshold. In the quantified proteins, the expression of 163 proteins in the HT/NC comparison group occurred up-regulation, and 45 proteins, down-regulated (Figure4. b). We used three statistical analysis methods, namely, principal component analysis (PCA), relative standard deviation (RSD) and Pearson's Correlation Coefficient, so that we could evaluate the quantitative repeatability of protein (Supplement Figure1. a, b, c).

We performed statistics on the distribution of differentially expressed proteins in GO secondary annotations. GO annotations are divided into three major categories: Biological Process, Cellular Component and Molecular Function, which explain the biological role of proteins from different perspectives. The results showed that the addition of hydroxytyrosol to the semen diluent made the protein expression different in Biological Process, Cellular Component and Molecular Function (Figure4. c; Supplement Figure2. a. b). Then, we used software to perform subcellular structure localization prediction and classification statistics for differentially expressed proteins (Figure4. d; Supplement Figure2. c. d). Through database comparison and analysis, COG (Clusters of Orthologous Groups of proteins) / KOG functional classification statistics of differentially expressed proteins were performed (Figure4. e; Supplement Figure2. e. f). The results showed that the differences were mainly observed in the protein translation modification, normal cell function, and signal transduction. Besides, these differential proteins also play an important role in maintaining the cytoskeleton and normal physiological function.

We performed enrichment analysis on three levels of GO classification, KEGG pathway, and protein domain for differentially expressed proteins in each comparison group (Figure5; Supplement Figure 3; Supplement Figure4). The purpose was to discover whether differentially expressed proteins had a significant enrichment trend in certain functional types. For the enrichment test (Fisher's exact test is used here), the *P*-value obtained by the bubble chart showed the functional classification and pathway of

significant enrichment of differential proteins ($P < 0.05$). We performed differential protein enrichment analysis on the three major categories (Biological Process, Cellular Component, Molecular Function) in GO classification (Supplement Figure5). The results of the data showed that the protein difference between the treated group and the control group was mainly reflected in the direction of membrane lipid transport and energy metabolism. The results of KEGG analysis were not highly correlated in sperm motility, but the protein difference in cell metabolism is surprising. The enrichment performance of protein domains showed differences in cell energy supply and cell metabolism.

The biological process results showed that the differential proteins played an important role in regulating gene expression and maintaining the normal cell cycle (Figure6. b). The results of cellular components showed that proteins were differentially expressed in maintaining cell membrane structure and mitochondrial function (Figure6. c). In terms of molecular function, these differential proteins had a wide influence on the enzymes involved in the redox process, which was beneficial to maintain the normal function of cells (Figure6. d). The results of KEGG pathway indicated that the differential protein expression mainly affected the direction of disease, but also had a significant role in maintaining cellular pathways and normal metabolism (Figure6. e) ($P < 0.05$). The results of the protein domain showed that the differential proteins were mainly concentrated in cellular immunity and transport, which was also very important for improving the quality of sperm during storage (Figure6. f).

The addition of hydroxytyrosol improves the sow's pregnancy rate and piglet's birth weight.

According to the results of artificial insemination in pigs (Table 2), the average pregnancy rate of the HT 120 μmol/L storage group reached 89.30%, which was significantly higher than the control group of 76.70% ($P < 0.01$). Besides, the preservation method of adding hydroxytyrosol not only significantly increased the pregnancy rate of the sow and the birth weight of the piglets, but also promoted the results of artificial insemination ($P < 0.05$).

Discussion

The concentration of ROS in pig semen at room temperature will accumulate over time, which is a major factor threatening the quality of pig semen [14, 26]. In our study, we found that the activity of pig sperm stored with hydroxytyrosol was better than control group from the second day of storage and the activity of sperm in the group of 120 μmol/L hydroxytyrosol was the best. Besides, the sperm motility was maintained at 61.09% on the fourth day, which greatly maintained the viability of the pig sperm. As one of the prerequisites for maintaining sperm motility and sperm-related physiological activities, low-dose ROS could help to improve sperm capacitation and protein phosphorylation [2]. However, high level of ROS could lead to male infertility. Many studies have demonstrated that antioxidants can reduce cellular ROS accumulation [17]. Semen ROS concentration was maintained at a lower level at the beginning of normal temperature storage, which is consistent with the results of Sutovsky's study [23]. However, with longer storage, especially in the later stage of preservation, the active oxygen content of semen increased exponentially. In addition, accompanied by a decrease in sperm quality and damage to the membrane.

This result indicates the rise of the active oxygen content of the semen causes a higher oxidative stress state of the sperm, the addition of hydroxytyrosol relieves the accumulation tendency of the active oxygen in the semen and reduces the accumulation of active oxygen. As the cumulative curve of the content of active oxygen in semen in 120 μmol/L of hydroxytyrosol was the slowest, we think it is also dose-dependent, the reason for which is still unknown. Subsequently, by further detecting the degree of damage of the plasma membrane and acrosome of the semen during storage, it was found that the plasma membrane suffered severe damage with longer storage, and the integrity of the plasma membrane decreased exponentially in the later stage of preservation. The detection of membranous peroxidation marker malondialdehyde showed a significant increase in the semen on the later stage of preservation and the peroxidation of the membrane of the sperm. The increase of the MDA content in the semen was inhibited after the addition of hydroxytyrosol and showed dose-dependence. Besides, plasma membrane was detected to be dose-dependent. However, it seemed that the integrity and status of the pig sperm acrosome were not affected by the dose during the entire preservation. Subsequently, on the fifth day of storage, the DNA damage of the hydroxytyrosol 120 μmol/L group and the control group was observed, showing that the control group's DNA was greatly fragmented and easily damaged, which indicated the accumulation of ROS. Meanwhile, this may cause oxidative damage to the sperm DNA, which is alleviated by the addition of hydroxytyrosol, and is consistent with Arando's findings on frozen sperm in sheep [3].

The antioxidant enzyme system of mammalian sperm consists of the following enzymes: SOD, GSH-Px, glutathione reductase and catalase [9], and some low molecular weight antioxidants, such as L-glutathione. Glycopeptide (GSH), L-ergothione (ERT), L-ascorbic acid, which were designed to neutralize sperm production of harmful active substances including superoxide (O^{2-}), hydroxyl (•OH) and Hydrogen peroxide (H_2O_2) [1]. At present, it is unclear whether hydroxytyrosol reduces the active oxygen content of semen by directly neutralizing the active oxygen in the semen or directly participating in the production of antioxidant enzymes. In the initial stage of semen preservation, the antioxidant enzyme activity in the semen was in the best state, and the total antioxidant capacity was highest. There was no significant difference between control group and hydroxytyrosol-added groups on day 0. The results showed that the activity of the antioxidant enzyme system decreased gradually. After adding hydroxytyrosol, the trend of the decrease in enzyme activity was delayed, and the state of the whole antioxidant enzyme system was improved. Especially, the addition of 120 μmol/L improved the sperm quality as well as its ability to remove ROS, and reduced the degree of damage. In addition, our result demonstrated that the hydroxytyrosol works more prominently in the middle and later stages of semen preservation.

The addition of hydroxytyrosol to pig semen dilutions greatly affected the expression of porcine sperm protein. In the subsequent artificial insemination test, the pregnancy rate of the sow and the newborn litter weight of the piglets were significantly increased ($P < 0.05$).

In actual production, the commonly used method of pig semen storage is short-term preservation [15]. When semen is stored after 72 hours, even if the accumulation of semen active oxygen in the short-term preservation process still exists, sperm damage is too enormous to be used.

The improvement of semen quality by adding hydroxytyrosol has been verified by artificial insemination. And the mechanism of hydroxytyrosol should be further explored in the near future.

Conclusion

One of the key problems to improve the preservation effect of boar semen at room temperature is to solve the accumulation of ROS. The results we reported here have demonstrated that the quality of boar sperm can be effectively improved by adding appropriate concentration of hydroxytyrosol into BTS diluent. In addition, adding hydroxytyrosol in BTS diluent can effectively reduce the damage of oxidative free radicals on sperm, which plays an important role in maintaining the normal physiological function of pig sperm. The results showed that the efficiency of artificial insemination was improved by adding appropriate amount of hydroxytyrosol, which was of great significance in practical production. In the future, the mechanism of improving the quality of pig sperm will have an important impact on artificial insemination technology.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of Northwest A&F University (Yangling Shaanxi, China). All operations were carried out according to the university's guidelines for animal research.

Consent for publication

All of the authors have approved the final version of the manuscript, agree with this submission to Journal of Animal Science and Biotechnology.

Availability of data and material

All the data analysed during this study are included in this article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Taiyong Yu is the leader of the project, conceived and designed the experiment. Wenyu Zhang and Dong Li performed experiments, analyzed data and wrote the manuscript. Xuekai Tian, Yulin He, Zitong Xiao and Xiaoting Wang contributed to the manuscript preparation. Renrang Du and Gongshe Yang contributed to the revisions. All authors reviewed and approved the final manuscript.

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Abbreviations

AI: Artificial insemination; HT: hydroxytyrosol; ROS: reactive oxygen species.

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Tables

Due to technical limitations, tables 1-2 are only available as downloads in the supplemental files section.

Figures

Fig. 1

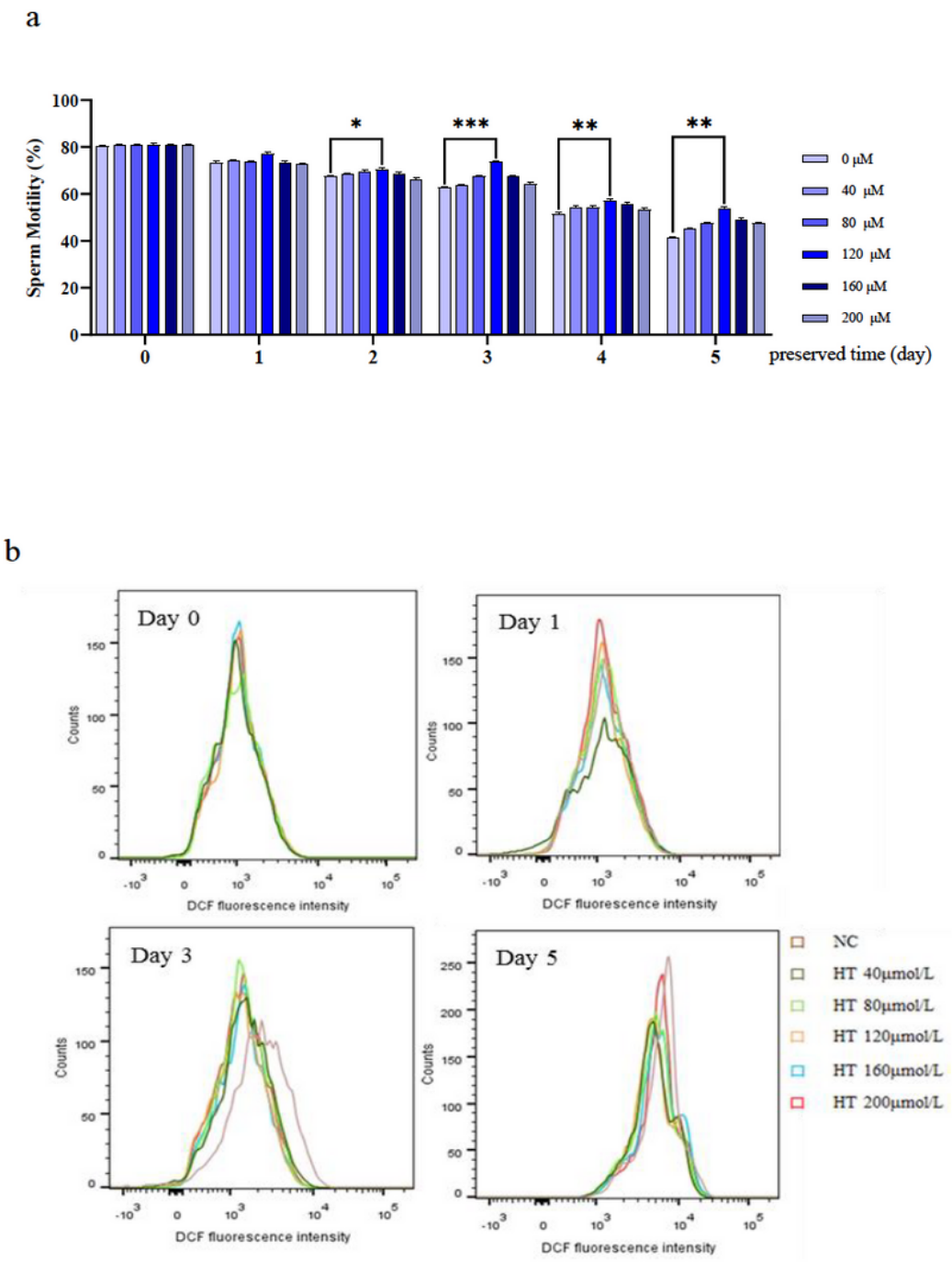

Figure 1

Hydroxytyrosol improves the preservation of pig semen at 17°C. a. Effect of adding different concentrations of hydroxytyrosol on the sperm motility of pigs (Note: * indicates P < 0.05, ** indicates P < 0.01, *** indicates P < 0.001). b. Fluorescence intensity of DCF in each test group at different storage times.

Fig. 2

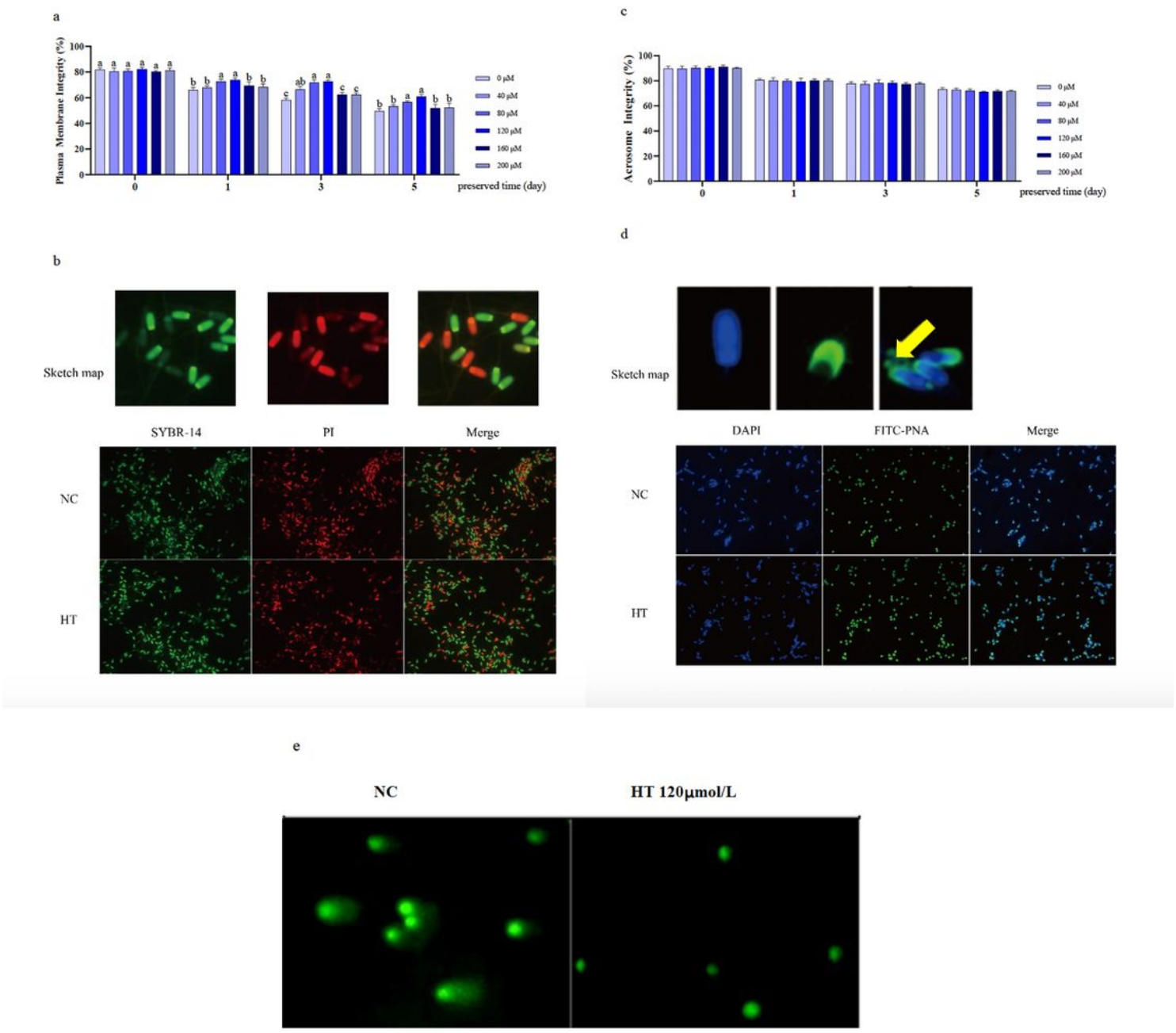

Figure 2

a. Effect of different concentrations of hydroxytyrosol on semen plasma membrane in semen dilution. b. Preservation of the complete acrosome of 120μmol/L hydroxytyrosol and control group on Day 3 (Note: SYBR-14 green fluorescent labeled spermatozoa with intact plasma membrane, PI red fluorescent labeled spermatozoa damaged by plasma membrane) (Scale: 50μm). c. Effect of adding different concentrations of hydroxytyrosol in semen dilution on the integrity of pig sperm acrosome. d. Preservation of the

complete acrosome of 120μmol/L hydroxytyrosol and control group on Day 3 (Note: DAPI blue fluorescent labeled spermatozoa with nucleus, FITC-PNA green fluorescent labeled spermatozoa with acrosome, Yellow arrow indicates damage to the acrosome) (Scale: 50μm). e. Semen DNA damage in 120 μmol/L hydroxytyrosol and control group when Semen was stored in Day 3. Note: The same letter indicates P > 0.05, different letters indicate P < 0.05.

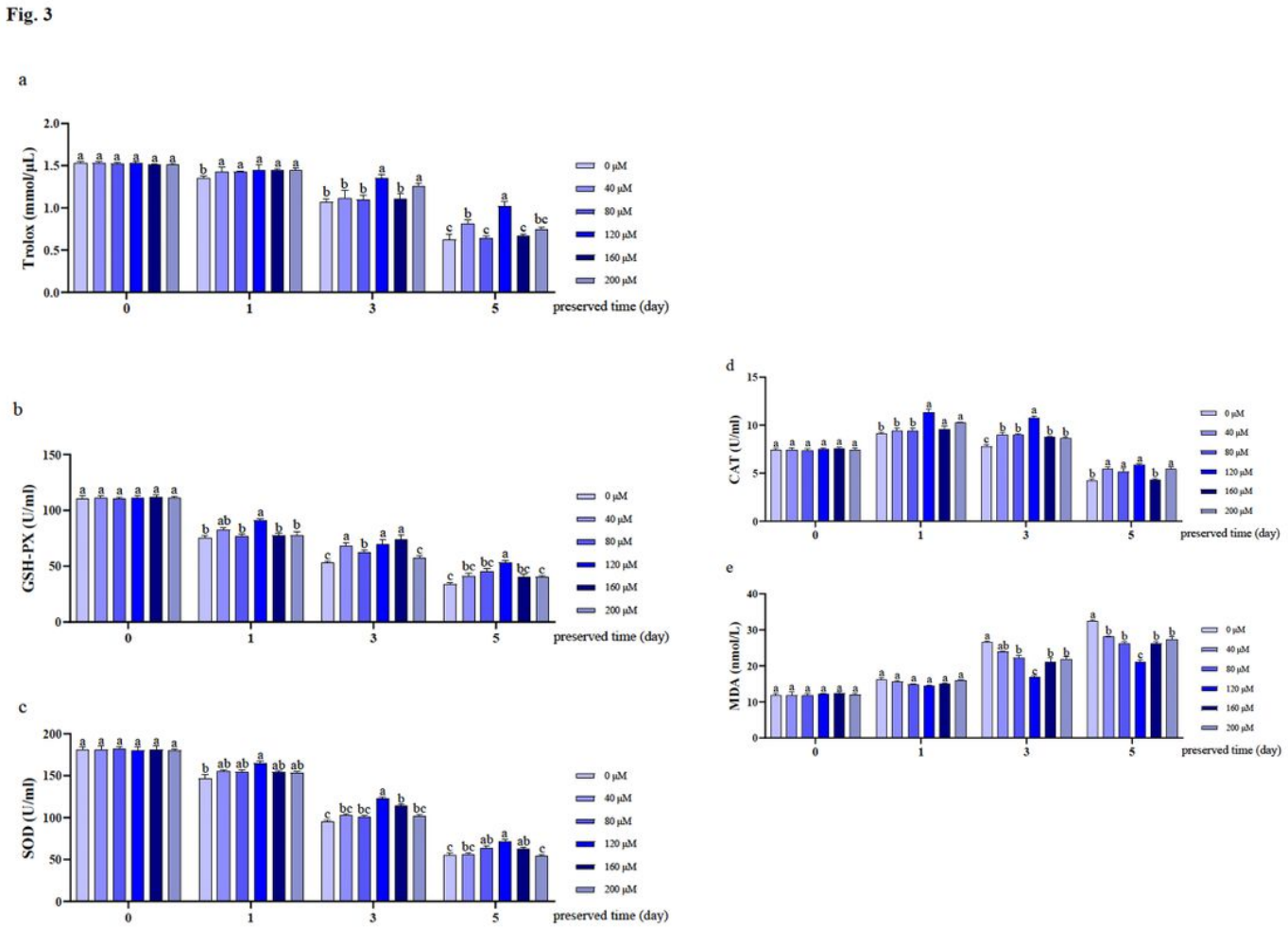

Figure 3

Hydroxytyrosol slows the rate of decline in sperm antioxidant enzyme activity. a. Effect of adding different concentrations of hydroxytyrosol on total antioxidant capacity of pig sperm. b. Effect of different concentrations of hydroxytyrosol on glutathione peroxidase in pig sperm. c. Effect of different concentrations of hydroxytyrosol on pig sperm superoxide dismutase. d. Effect of adding different concentrations of hydroxytyrosol on pig sperm catalase. e. Effect of adding different concentrations of hydroxytyrosol on the accumulation of malondialdehyde in pig sperm. Note: The same letter indicates P > 0.05, and different letters indicate P < 0.05.

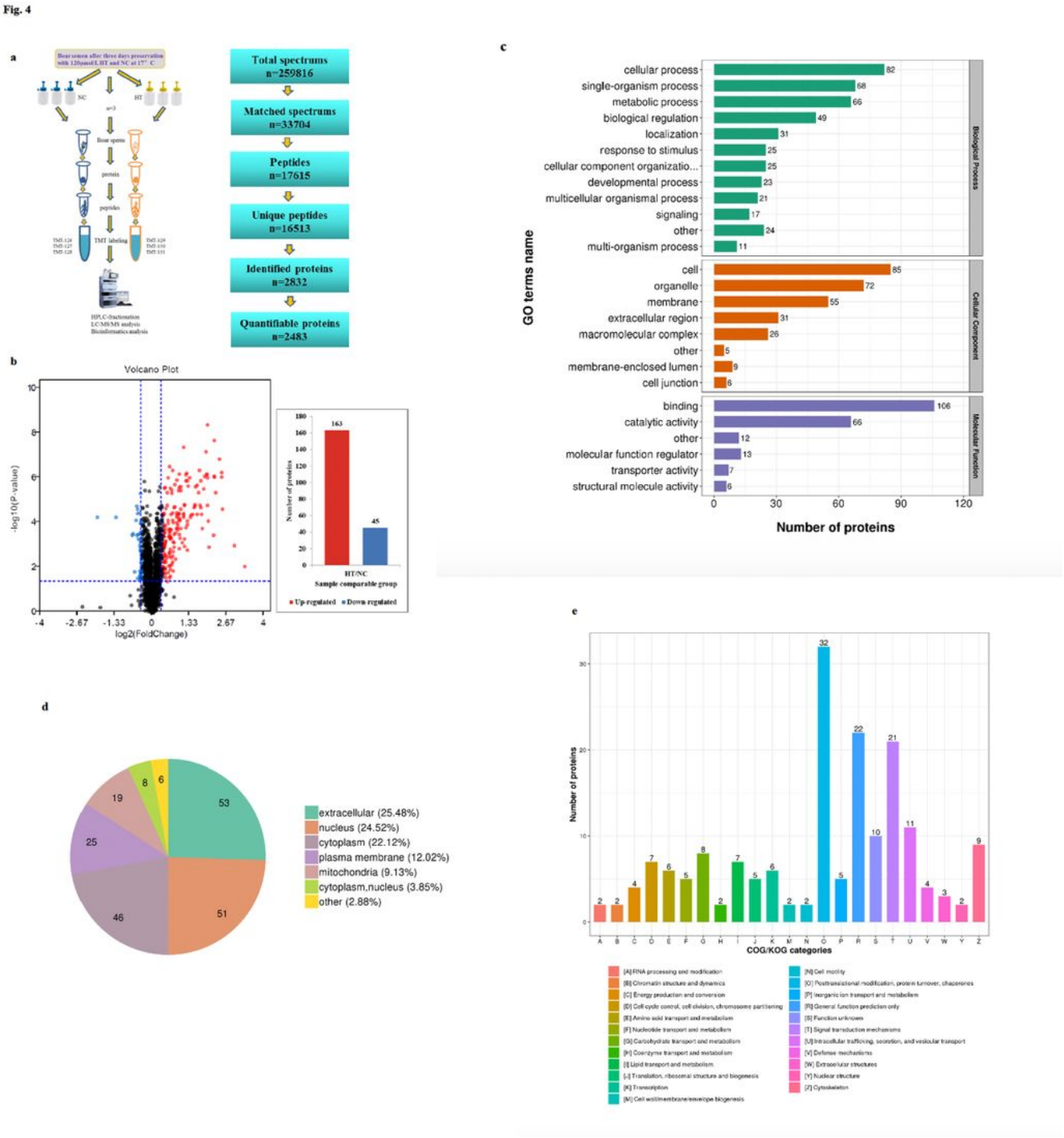

Figure 4

a. Proteomics analysis process and Statistical results of protein mass spectrometry. b. Volcanic map of differentially expressed protein expression in HT/NC. c. Statistical distribution chart of Differentially expressed proteins under each GO category (2nd Level). d. Subcellular localization chart of Differentially expressed proteins. e. COG/KOG functional classification chart of Differentially expressed proteins.

Fig. 5

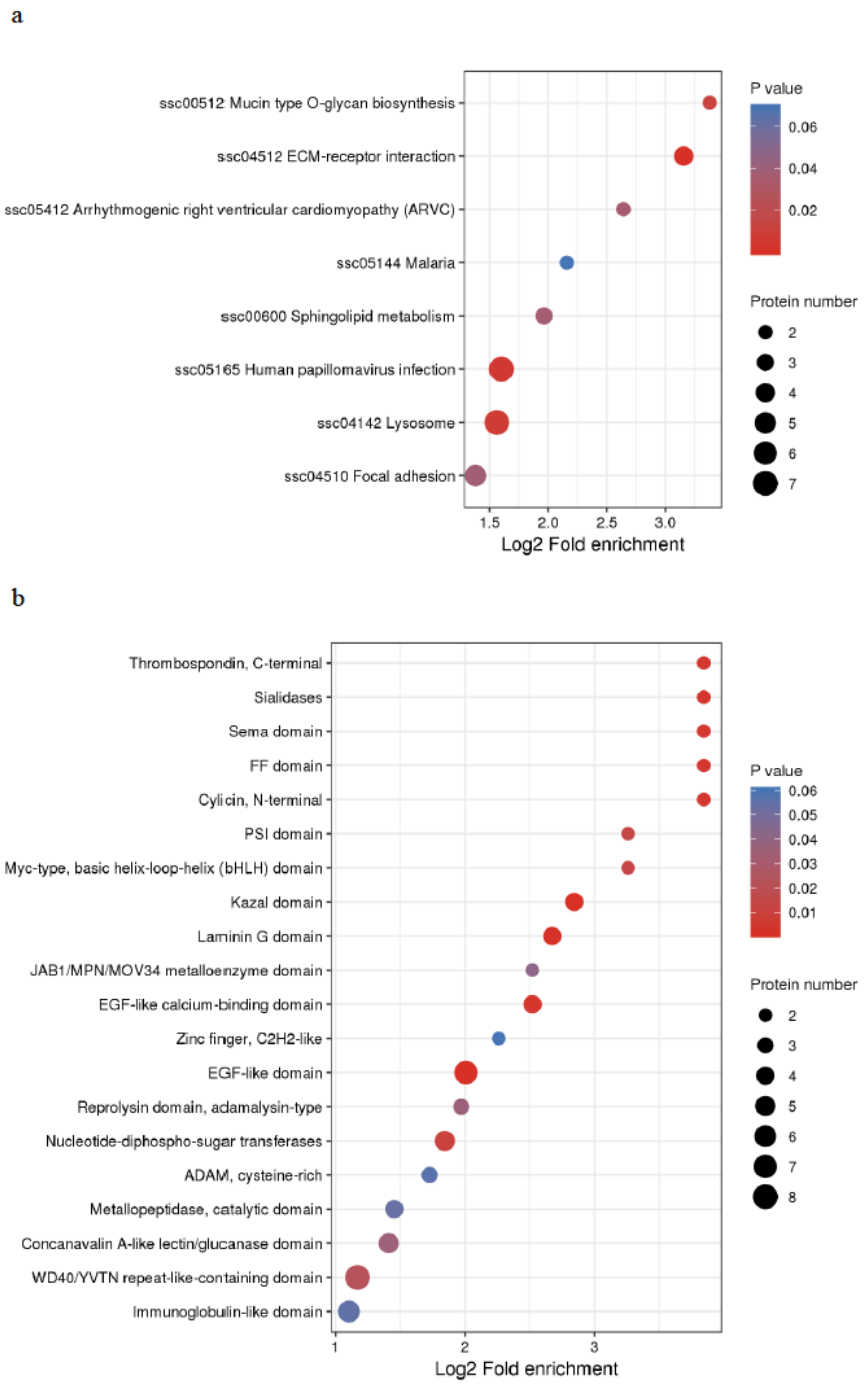

Figure 5

a. KEGG pathway enrichment bubble plot of Differentially expressed proteins. b. Protein domain enrichment bubble plot of Differentially expressed proteins. The bubble chart gives the results of the top 20 classifications with the most significant enrichment. In the bubble chart, the vertical axis is the functional classification or pathway, and the horizontal axis value is the log2 converted value of the fold change of the proportion of the difference protein in the function type compared with the proportion of

the identified protein. The color of the circle indicates the enrichment significance p-value, and the size of the circle indicates the number of differential proteins in functional classes or pathways.

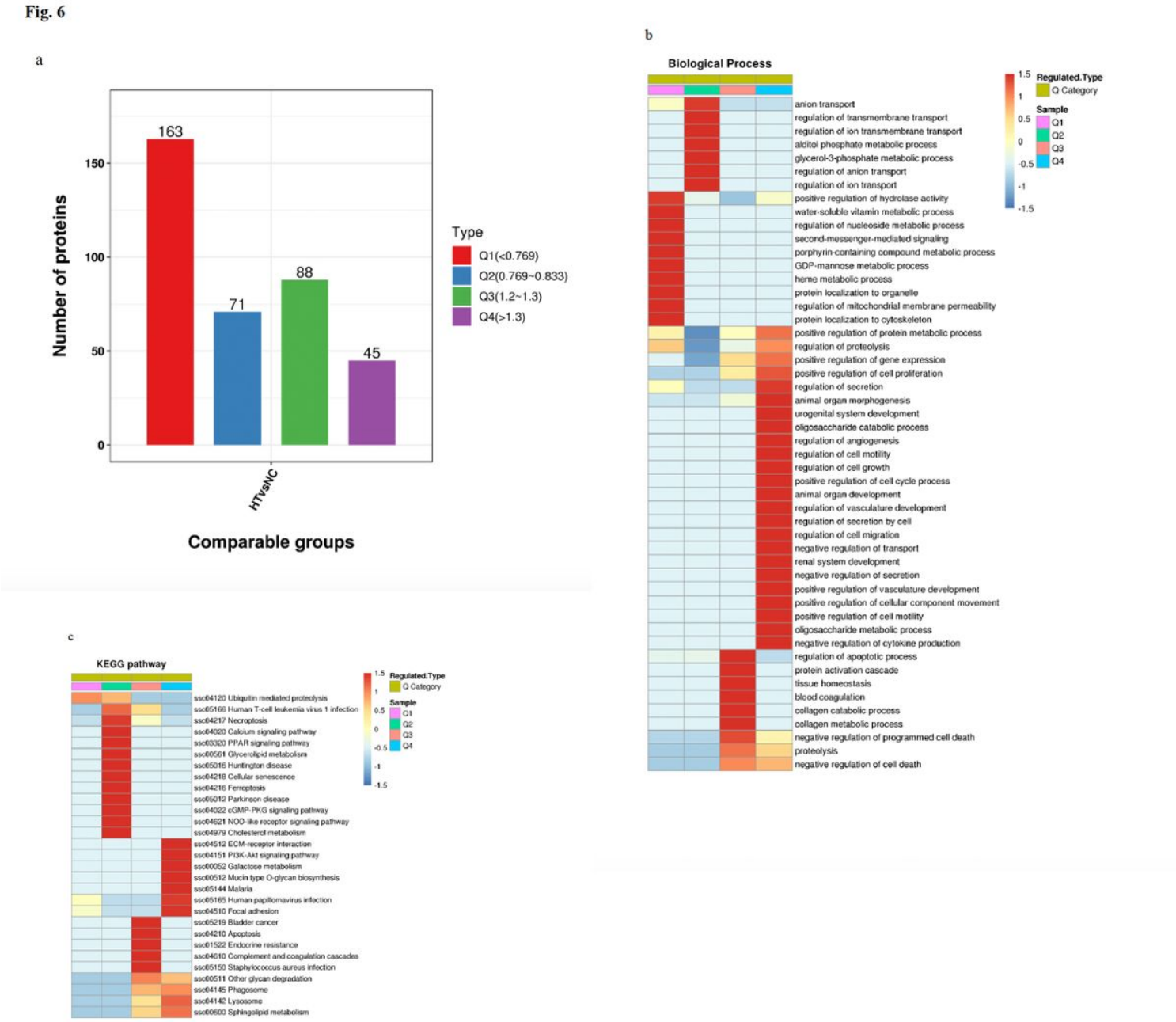

Figure 6

For the differentially expressed proteins, we divided them into 4 parts according to their differential expression multiples, called Q1 to Q4. According to the P value of enrichment analysis (Fisher's exact test) obtained by enrichment analysis, hierarchical clustering method is used to group related functions in different groups together and draw as a heatmap. The horizontal direction of the heat map represents the enrichment test results of different groups, and the vertical direction describes the functions of

differential expression enrichment (GO, KEGG pathway, protein domain). The color blocks corresponding to the differentially expressed proteins and function descriptions of different groups indicate the degree of enrichment. Red represents a strong degree of enrichment, and blue represents a weak degree of enrichment. a. The difference protein is divided into Q1-Q4 number distribution according to the multiple. b. Comprehensive heatmap for cluster analysis of the enrichment patterns of GO functional categories (Biological process). c. Comprehensive heatmap for cluster analysis of the enrichment patterns of KEGG pathways.

Supplementary Files

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