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Short communication

Identification and characterisation of mitochondrial proteins isolated from rabbit epididymal spermatozoa – a preliminary study

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Abstract

This is the first study to identify 23 protein spots corresponding to 13 proteins in mitochondria isolated from rabbit epididymal spermatozoa. In the group of protein spots identified in stress-induced samples, the abundance of 20 protein spots increased, whereas the abundance of three protein spots (GSTM3, CUNH9orf172, ODF1) decreased relative to the control. The results of this study provide valuable inputs for future research into the molecular mechanisms implicated in pathological processes during oxidative stress (OS).

Keywords: spermatozoa, mitochondria, oxidative stress, proteins profile

Introduction

Rabbits are valuable animals in both livestock farming and biomedical sciences. The male rabbit is a suitable animal model for research in reproductive biology due to its size, low cost of maintenance, and relative simplicity and effectiveness of collecting semen.

Artificial insemination (AI) with fresh, chilled and/or cryopreserved semen is a routine practice in the rabbit industry, and the influence of environmental factors and semen collection and preservation methods on the quality of spermatozoa and breeding success has been

extensively researched (Abdelnour et al. 2020, Halo et al. 2021, Laghouati et al. 2021, Nishijima et al. 2021). Casares-Crespo et al. (2018, 2019) relied on proteomic methods to identify and characterise seminal plasma proteins and spermatozoa proteins, and to determine differences in their genetic origin. Bezerra et al. (2019) described the major seminal plasma proteins and their potential associations with semen quality.

Sperm preservation techniques can lead to plasma membrane damage, motility disorders, and chromatin destabilisation due to, among other factors, excessive production of reactive oxygen species (ROS) (Aitken

Table 1. Mitochondrial proteins isolated from rabbit epididymal spermatozoa. Proteins whose expression decreased under experimental conditions (relative to control conditions) are marked in bold font, and proteins whose expression increased are marked in normal font.

No	Protein name (gene)	NCBI database index number	Calculated MW/PI	Mascot score
1	E3 ubiquitin-protein ligase ARIH2 isoform X4 (ARIH2)	XP_017199297.1	43230/7.93	83
2	Mitochondrial ATP synthase subunit beta (ATP5B)	XP_002711130.3	56321/5.15	362
3	Mitochondrial heat shock protein 60 kDa (HSPD1)	XP_002712414.1	61174/5.70	280
4	Glucose-regulated protein 78 kDa (HSPA5)	XP_017205553.1	72340/5.07	140
5	Glucose-regulated protein 78 kDa (HSPA5)	XP_017205553.1	72340/5.07	116
6	Glutathione S-transferase Mu 3 (GSTM3)	XP_002715838.3	27147/5.99	163
7	Serum albumin, Chain A (PDBe)	3V09	67986/5.65	124
8	Serum albumin, Chain A (PDBe)	3V09	67986/5.65	124
9	Epididymal protein 38kDa (rep38-c1)	AAC95386.1	77808/6.10	169
10	zonadhesin isoform X1 (ZAN)	XP_008256210.1	281757/6.10	86/4
11	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific isoform X2 (GAPDHS)	XP_002722284.1	44211/7.07	127
12	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific isoform X2 (GAPDHS)	XP_002722284.1	44211/7.07	127
13	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific isoform X2 (GAPDHS)	XP_002722284.1	44211/7.07	127
14	Precursor angiotensin-converting enzyme isoform 2 (ACE2)	NP_001164540.1	84383/6.69	195
15	Precursor angiotensin-converting enzyme isoform 2 (ACE2)	NP_001164540.1	84383/6.69	195
16	Precursor angiotensin-converting enzyme isoform 2 (ACE2)	NP_001164540.1	84383/6.69	195
17	Precursor angiotensin-converting enzyme isoform 2 (ACE2)	NP_001164540.1	84383/6.69	195
18	Precursor angiotensin-converting enzyme isoform 2 (ACE2)	NP_001164540.1	84383/6.69	195
19	Uncharacterized protein C9orf9 homolog isoform X2 (CUNH9orf172)	XP_017193431.1	19690/8.21	199/2
20	Outer dense fiber protein 1 (ODF1)	XP_002710767.1	30706/8.44	103
21	L-amino-acid oxidase, partial (L-AOX)	XP_017195962.1	63834/9.33	402
22	L-amino-acid oxidase, partial (L-AOX)	XP_017195962.1	63834/9.33	402
23	ATP synthase subunit alpha, mitochondrial (ATP5F1A)	XP_002713558.1	59831/9.15	96

et al. 2012). However, spermatozoa's ability to precisely localise and control the generation of small amounts of ROS is essential for the capacitation process, acrosome reaction, or redox signalling that determines fertilisation success (Agarwal et al. 2014).

Due to the growing interest in oxidative stress (OS) in sperm cells and the general scarcity of the relevant research, this study was undertaken to characterise the protein profile of mitochondria isolated from rabbit epididymal spermatozoa under physiological conditions and after the induction of OS.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Testes with epididymides were harvested from four

routinely slaughtered mature male rabbits (Flemish Giant rabbit, *Oryctolagus cuniculus domesticus*) aged 2 years. The obtained material was transported within one hour to the laboratory at 4°C. Upon delivery, gonads were immediately rinsed with PBS, and epididymal spermatozoa were aspirated from the cauda epididymis with a needle. Each aspirated sperm cell was divided into two equal aliquots, twice rinsed with PBS, and centrifuged at 350×g/10 min/4°C. Subsequently, the supernatant was discarded, and the control samples and the experimental samples were resuspended in 500 µl PBS and 500 µl PBS, respectively, with the addition of 50 mM menadione, and incubated at room temperature for one hour. Mitochondria were isolated using a commercial kit (Mitochondria Isolation Kit for Mammalian Cells, Thermo Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions. Pellets containing the isolated mitochondria were resus-

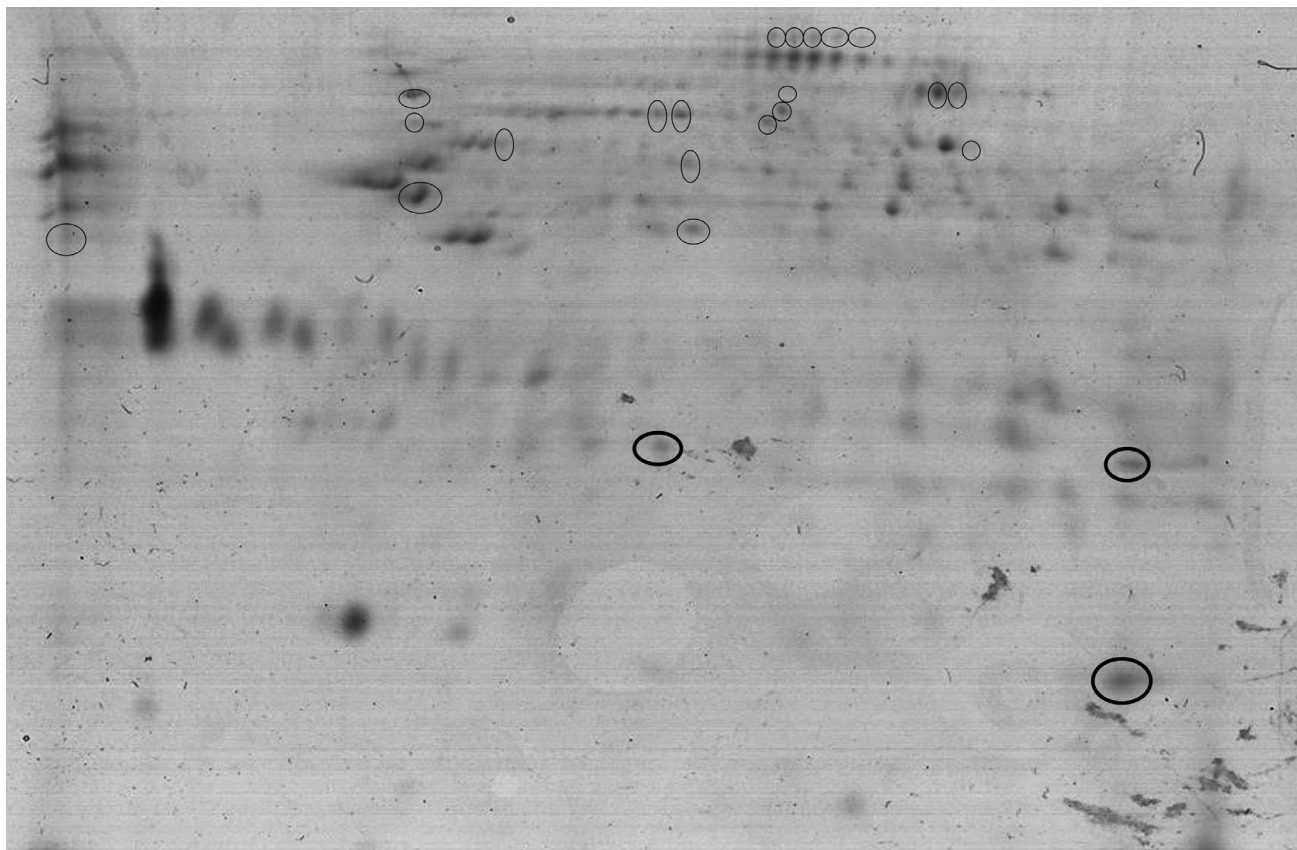


Fig. 1. 2D gel images of representative proteins extracted from the mitochondria of rabbit epididymal spermatozoa. Proteins whose expression decreased under experimental conditions (relative to control conditions) are marked in bold font, and proteins whose expression increased are marked in normal font.

pended in 80 μ l of a lysis buffer (7M urea, 2M thiourea, 4% [wt/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS]), 2.5% (v/v) protease inhibitor cocktail, 1% (v/v) phosphate inhibitor cocktail, 0.1 mM neocuproine), sonicated on ice, and centrifuged at 1000 \times g/30 min/18°C. The pellets were then resuspended in a rehydration buffer (7M urea, 2M thiourea, 4% CHAPS). Protein concentration was measured using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples containing 50 μ g of protein were loaded onto 10-cm non-linear Immobiline DryStrips with a pH of 3 to 10 (GE Healthcare, Chicago, IL, USA). After rehydration, proteins were separated by isoelectric focusing and subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) according to the method described by O'Farrell (1975) with some modifications. Gels were stained with Coomassie Brilliant Blue R-250 and analysed with SameSpots software (TotalLab, Newcastle upon Tyne, England, UK). Proteins characterised by changes in spot intensity ($p < 0.05$, fold change > 2) were selected for further identification by mass spectrometry, which was conducted according to the protocol described by Majewska et al. (2017) with some modifications.

Results and Discussion

This is the first study to identify 23 protein spots corresponding to 13 proteins in mitochondria isolated from rabbit epididymal spermatozoa (Table 1). In the group of protein spots identified in stress-induced samples, the abundance of 20 protein spots increased, whereas the abundance of three protein spots decreased relative to the control (Fig. 1).

The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme which plays numerous roles in various biological processes (Hancock et al. 2020), increased under OS conditions. The analysis also revealed an increase in the expression of protein chaperones such as mitochondrial heat shock protein 60kDa and glucose-regulated protein 78kDa, which participate in protein folding and assembly in the refolding process of denatured polypeptides to prevent the formation of aggregates (Walsh et al. 2008). The expression of mitochondrial ATP synthase, which is involved in oxidative phosphorylation and ATP synthesis via a proton gradient (Walker et al. 2013), also increased during OS. Interestingly, the expression of glutathione S-transferase Mu 3, which participates in cell detoxification and signalling regulation (Raza

et al. 2011), decreased. The results of this study provide valuable inputs for future research into the molecular mechanisms implicated in pathological processes during OS.

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