**Age-associated metabolomic changes in human spermatozoa**

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Advancing age is associated with a significant decline in fertility in men. Globally, aging associated reproductive health and wellbeing are assuming considerable importance as lifestyle changes have led to delayed childbearing and an increasing number of advanced age couples seeking fertility treatment. For example, the number of men between 35 and 54 years of age fathering children has increased 25% since 1980 in the United States. Understanding age-related changes in spermatozoa is crucial for addressing fertility challenges in aging men. Very limited attention has been focused on age-related functional genomics, especially metabolomic changes in human spermatozoa. The present study addressed this issue. The study involved 18 fertile Arab/Saudi men aged 21 to 51 years. The participants underwent clinical assessments at King Abdulaziz University Hospital, Jeddah, and were categorized into three age groups: young adult (21–30 years; n = 6), late adult (31–40 years; n = 7), and advanced age (41–51 years; n = 5). Semen samples were gradient purified, and purified spermatozoa were subjected to untargeted metabolomic analysis using LC-MS/MS. The spectral data were processed using XCMS and the metabolites were identified using the Human Metabolome Database (HMBD). Statistical and differential analyses were performed using MetaboAnalyst. Mean (±SD) ages among the three groups were different (27.8±2.6 years, 34.3±2.9 years, and 47.2±4.2 years; $P<0.05$). Mean sperm concentrations were higher ($P<0.05$) in late adult group (85.4±22.0 M/ml) than young adult group (55.3±25.5 M/ml) but comparisons for the advanced age group (78.0±38.2 M/ml) were not significant. No significant differences were found in other semen analysis endpoints. Metabolomic analysis revealed 469 peak intensities on LC-MS/MS in the three groups. From these peaks, 380 metabolites were identified through HMDB, whereas 89 peak intensities could not be identified. The principal component analysis of 380 metabolites showed distinct separate clustering of metabolite expression for young adult and advanced age groups whereas the data for late adult group aligned intermediately with both groups. The ANOVA analysis showed significant differences ($P<0.05$) in 167 metabolites among the three age groups. Using a fold-change threshold of ≥ 2, pair-wise analysis showed differential abundance of 31 metabolites in young adult vs. late adult groups (23 upregulated, 8 down regulated), 179 metabolites in young adult vs. advanced age

groups (96 upregulated, 83 down regulated), and 39 metabolites in late adult vs. advanced age groups (16 upregulated and 23 down regulated). Comparing the young adult group with advanced age group, 179 metabolites exceeded variable importance in projection (VIP) score $\geq 1$ indicating their significant contribution to group differentiation. Among these top VIP scored metabolites, L-homocysteine, L-cysteine, (S)-hydroxyoctanoyl-CoA, glutathione, and acetyl CoA were downregulated, and HMG-CoA, pentadecanoyl-CoA, CL(22:6/20:4/22:6/22:6), PIP(TXB2/22:3(10Z,13Z,16Z)), and uracil were upregulated in the advanced age group. Of note was almost complete absence of homocysteine in the spermatozoa of all the men in advanced age group. These deregulated metabolites are involved in crucial processes of sperm physiology including energy metabolism, sperm development and function, mitochondrial integrity and function, antioxidation, cholesterol metabolism, *etc.* In conclusion, dynamic metabolomic changes in advancing age potentially impact crucial human sperm physiological functions affecting normal sperm fertility.

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