Abstract

Scientific Background: Reproductive management in both domestic and global dairy cattle farming is heavily reliant on artificial insemination and characterized by the utilization of cryopreserved semen. Ejaculate quality serves as a major determinant in this intensive reproductive management system. A decline or impairment in semen quality during cryopreservation processes can significantly impact fertilizing capacity and conception rates. Semen quality can be evaluated via physiological and biochemical parameters, as well as the correlations between them. The number of spermatozoa exhibiting progressive motility (straight-line forward movement) is widely utilized as an index to determine semen quality. Recent studies indicate the involvement of the endocannabinoid system (ECS) in numerous pathways associated with the male reproductive system. Arachidonic acid (AA)—a polyunsaturated fatty acid (PUFA) that plays a key role in structural cell membrane assembly—serves as both the precursor molecule and the enzymatic degradation product of the primary endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG). In a previous study, we demonstrated a relationship between the concentration of PUFAs in spermatozoa and progressive cell survival. Accordingly, it can be hypothesized that a link exists between the endocannabinoid system, cell membrane composition, and sperm function. In the current study, we investigated the relationship between progressive motility patterns and ECS components within bull spermatozoa and seminal plasma.

Research Aims: The general objective of this study was to examine the relationship between progressive sperm motility and the endocannabinoid system. The specific aims were:

1. To characterize the endocannabinoid profile in seminal plasma and determine whether a correlation exists between this profile and progressive motility patterns.
2. To characterize the presence of cannabinoid receptors (CB1, CB2, TRPV1) and the fatty acid amide hydrolase (FAAH) enzyme in spermatozoa, and to assess whether a relationship exists between these components and progressive motility patterns.
3. To evaluate the relationship between ECS components and progressive motility survival.

Experimental Design

During the first year of the study, 15 ejaculates were collected from different bulls in accordance with the seasonal breeding schedule. Ejaculates (5 per group) were allocated into three groups based on their baseline progressive motility patterns relative to the database at that time:

- High Progressive Motility (HPM): >74\% progressively motile cells.
- Medium Progressive Motility (MPM): $60\% - 74\% progressively motile cells.
- Low Progressive Motility (LPM): < 59\% progressively motile cells.

Each ejaculate was centrifuged to separate the cellular fraction (spermatozoa) from the liquid fraction (seminal plasma). For each isolated sample, seminal plasma and spermatozoa were processed for fatty acid and endocannabinoid profiling. Additionally, an aliquot from each ejaculate was taken to evaluate the survival rate of progressively motile cells. Extraction, identification, and quantification of fatty acids in seminal plasma and spermatozoa were performed following lipid fraction extraction using gas chromatography. During the second and third years of the study, ECS components were analyzed by evaluating the protein expression of CB1, CB2, TRPV1, FAAH, DAGLB, and NAPE-PLD in spermatozoa. Statistical analyses were performed using IBM SPSS Statistics software (v23.0). Two-tailed Spearman non-parametric correlation analyses were conducted across the total sample and within each experimental group (HPM, MPM, LPM) to evaluate relationships between ECS components in both seminal plasma and spermatozoa. Differences in endocannabinoid profiles between progressive motility groups were analyzed using one-way ANOVA, followed by Tukey's HSD post-hoc test for pairwise comparisons. Inter-group differences were further analyzed using independent samples t-tests ($P < 0.05$). Protein expression data were analyzed using SAS GLM software (2002).

Results: The proportion of morphologically normal cells and progressively motile cells was significantly lower in the LPM group compared to the HPM and MPM groups ($P < 0.05$). The overall percentage of motile cells was higher in the HPM group compared to the LPM group ($P < 0.05$). The percentages of oleic acid (C18:1n9), vaccenic acid (C18:1n7), and linoleic acid (C18:2n6) were significantly lower in the HPM group within both spermatozoa and seminal plasma. No significant differences were observed in overall fatty acid classes (saturated, unsaturated, omega-3, and omega-6 polyunsaturated fatty acids) among the HPM, MPM, and LPM groups in spermatozoa. The proportion of arachidonic acid (C20:4n6), which serves as both a biosynthetic precursor and an enzymatic degradation product of endocannabinoids, was lower in both spermatozoa and seminal plasma of the HPM group compared to the LPM group. While no significant inter-group differences were found regarding docosahexaenoic acid (DHA, C22:6n3) content in spermatozoa, the percentage of DHA was higher in the seminal plasma of HPM samples compared to LPM samples. Furthermore, the total proportion of monounsaturated and polyunsaturated fatty acids was lower in the seminal plasma of HPM cells compared to MPM cells. Omega-6 fatty acid levels were lower in the seminal plasma of the HPM group compared to the MPM group ($P < 0.05$); however, no differences in omega-3 fatty acid levels were detected between groups. Regarding the relative endocannabinoid profile, 2-AG levels were significantly lower in the seminal plasma of HPM samples compared to MPM samples. AEA was not detected in seminal plasma. In the third year of the study, an absolute quantitative analysis of endocannabinoid levels was performed at Prof. Joseph Tam's laboratory at the Hebrew University. AEA remained undetected in seminal plasma; it was found at low levels in spermatozoa, but no significant differences in sperm AEA levels were observed between the progressive motility groups. Correlation analysis within the seminal plasma fraction revealed no correlation between endocannabinoid levels, nor any significant associations with progressive motility survival. Intra-group correlation analyses within each individual motility group (HPM, MPM, LPM) showed no statistically significant correlations among the tested parameters in seminal plasma. In contrast, correlation analysis within the cellular fraction (spermatozoa) indicated significant positive correlations between molecules of the N-acylethanolamine family (AEA, OEA, and PEA) and progressive survival. Disaggregated intra-group correlation analyses demonstrated strong positive correlations between N-acylethanolamine family molecules specifically within the HPM group, whereas no statistically significant associations were found within the MPM and LPM groups. When assessing the impact of semen motility on sperm ECS protein expression, the expression of the monoacylglycerol lipase (MGLL) enzyme—which degrades the endocannabinoid 2-AG—was significantly lower in spermatozoa with low motility compared to those with high motility ($P = 0.045$). Conversely, the expression of diacylglycerol lipase beta (DAGLB)—the enzyme responsible for synthesizing 2-AG—was higher in low-motility cells compared to high-motility cells ($P = 0.0002$). No differences were observed between groups regarding the expression of CB1, CB2, or TRPV1 receptors, nor the enzymes NAPE-PLD and FAAH.

Discussion: Evaluation of fatty acid levels and endocannabinoid derivatives revealed several noteworthy findings. First, regarding arachidonic acid (AA, C20:4n6), which acts as a precursor for the synthesis of arachidonoylglycerol-type endocannabinoids, reduced AA levels were observed in both the cellular and seminal plasma fractions isolated from high progressive motility ejaculates. This HPM group was concurrently characterized by low levels of the endocannabinoid 2-AG. The low level of AA identified via quantitative endocannabinoid analysis in the seminal plasma of the HPM group supports the hypothesis that precursor availability for ECS components is linked to spermatozoal function. Conversely, and in contrast to our Year 1 findings, the lack of differences in AA levels within the spermatozoa themselves may indicate that the concentration of arachidonic acid in the extracellular environment (seminal plasma), rather than the intracellular environment, serves as the linking factor between the endocannabinoid system and progressive motility. In HPM semen samples, a lower percentage of linoleic acid (C18:2n6), the metabolic source for arachidonic acid synthesis, was found in both sperm cells and seminal plasma. Furthermore, the overall proportion of omega-6 fatty acids was lower in HPM bulls. It can therefore be hypothesized that the lower availability of omega-6 fatty acids in the cells and seminal plasma of HPM bulls is functionally related to the low levels of the endocannabinoid 2-AG. Linoleic acid (C18:2n6) also serves as a precursor for the endocannabinoid linoleoyl-ethanolamide (LEA), the levels of which were highest in the MPM group, intermediate in the HPM group, and lowest in the LPM group. Because LEA can bind to cannabinoid receptors, we hypothesize that LEA represents a potential endocannabinoid candidate associated with the degree of semen motility. Another finding concerns myristoyl-ethanolamide, an endocannabinoid-family molecule synthesized from myristic acid (C14:0) and ethanolamine. In this study, semen from the high progressive motility group was characterized by high levels of myristic acid (in both cells and seminal plasma) and a high presence of myristoyl-ethanolamide in the seminal plasma compared to semen with reduced progressive motility. This suggests that this molecule may represent a potential biomarker associated with sperm motility; however, follow-up studies are required to validate this hypothesis. Furthermore, we confirmed that bull spermatozoa express ECS components in line with current literature, and we characterized for the first time at the protein level the expression of the primary receptors (CB1, CB2, TRPV1) and key enzymes (NAPE-PLD, FAAH, MGLL, and DAGLB) in Israeli bulls. Interestingly, the levels of DAGLB and MGLL enzymes, which regulate 2-AG levels via synthesis and degradation respectively, differed between motility groups in an inversely proportional manner. DAGLB protein expression was lowest in cells with high progressive motility (HPM) and highest in those with low motility (LPM). In contrast, MGLL protein levels were lowest in the LPM group. We suggest that the expression of these enzymes, alongside fatty acid composition, can modulate the endocannabinoid profile within spermatozoa, and that these findings point to the potential involvement of the ECS in bovine progressive motility patterns. The finding that AEA was undetected in seminal plasma, alongside its presence at low levels within spermatozoa, may reflect the nature of this molecule as a short-range endocannabinoid subject to rapid degradation, primarily via the FAAH enzyme. The absence of correlation between ECS components in seminal plasma, as well as the lack of association with progressive survival, may indicate that seminal plasma ECS components do not necessarily directly reflect the metabolic dynamics linked to sperm motor function—at least for the molecules evaluated in this study. It is also possible that component concentrations in seminal plasma are influenced by confounding factors unrelated to progressive motility.

In conclusion, this study demonstrates for the first time a relationship between progressive motility patterns in bull spermatozoa and components of the ECS. The ECS within spermatozoa appears to feature a coordinated metabolic organization, whereas the seminal plasma may be subject to additional regulatory factors not directly coupled to progressive motility. It should be noted that intra-group analyses were conducted on small sub-samples; hence, these findings must be interpreted with caution. In light of these results, future studies should continue to elucidate the mechanisms linking the ECS to sperm motility, and consequently investigate ECS modulation strategies as an innovative approach to enhance semen quality in bulls.