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ORIGINAL ARTICLE *Andrology*

Microfluidic sorting selects sperm for clinical use with reduced DNA damage compared to density gradient centrifugation with swim-up in split semen samples

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STUDY QUESTION: Does microfluidic sorting improve the selection of sperm with lower DNA fragmentation over standard density-gradient centrifugation?

SUMMARY ANSWER: Microfluidic sorting of unprocessed semen allows for the selection of clinically usable, highly motile sperm with nearly undetectable levels of DNA fragmentation.

WHAT IS KNOWN ALREADY: Microfluidic devices have been explored to sort motile and morphologically normal sperm from a raw sample without centrifugation; however, it is uncertain whether DNA damage is reduced in this process.

STUDY DESIGN, SIZE, DURATION: This is a blinded, controlled laboratory study of differences in standard semen analysis parameters and the DNA fragmentation index (DFI) in split samples from infertile men ($n = 70$) that were discarded after routine semen analysis at an academic medical center.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Sperm concentration, progressive motility and forward progression were assessed by microscopic examination. For each sample, the unprocessed semen was tested for DNA fragmentation and split for processing by density-gradient centrifugation with swim-up or sorting by a microfluidic chip. DNA fragmentation was assessed in unprocessed and processed samples by sperm chromatin dispersion assay. The DFI was calculated, from up to 300 cells per slide, as the number of cells with fragmented DNA divided by the number of cells counted per slide.

MAIN RESULTS AND THE ROLE OF CHANCE: The median DFI in unprocessed samples was 21% (interquartile range (IQR): 14–30). In paired analyses of all samples, those processed by the microfluidic chip demonstrated significantly decreased DFI compared to those processed by density-gradient centrifugation ($P = 0.0029$) and unprocessed samples ($P < 0.0001$). The median DFI for chip specimens was 0% (IQR: 0–2.4) while those processed by density-gradient centrifugation had a median DFI of 6% (IQR: 2–11). Unprocessed samples in the highest DFI quartile (DFI range: 31–40%) had a median DFI of 15% (IQR: 11–19%) after density-gradient centrifugation and DFI of 0% (IQR: 0–1.9%) after processing with the microfluidic chip ($P = 0.02$).

LIMITATIONS, REASONS FOR CAUTION: While a high DFI has been associated with poor outcomes with IVF/ICSI, there are limited data illustrating improvements in clinical outcomes with a reduction in DFI. As this study utilized discarded, non-clinical samples, clinical outcomes data are not available.

WIDER IMPLICATIONS OF THE FINDINGS: While microfluidic sorting of unprocessed semen allowed for the selection of clinically usable, highly motile sperm with nearly undetectable levels of DNA fragmentation, standard processing by density-gradient centrifugation with swim-up did not increase DNA fragmentation in an infertile population. The proposed microfluidic technology offers a flow-free approach to sort sperm, requiring no peripheral equipment or filtration step, while minimizing hands-on time.

STUDY FUNDING/COMPETING INTEREST(S): No external funding to declare. Utkan Demirci, PhD is the Co-founder and Scientific Advisor for DxNow Inc., LevitasBio Inc. and Koek Biotech. Mitchell Rosen, MD is a member of the Clinical Advisory Board for DxNow Inc.

Key words: microfluidics / sperm / DNA fragmentation / density-gradient centrifugation / swim-up

Introduction

Male factor infertility is common, but poorly understood. The conventional semen analysis does not reliably predict fertility or treatment response, suggesting that sperm production is only part of the problem (Barratt *et al.*, 2010). In order to investigate other aspects in which sperm can impact embryonic development, assays for assessment of sperm DNA integrity have been sought to better understand how sperm quality may predict male fertility.

Sperm DNA fragmentation is increased in infertile men with abnormalities in standard semen analysis parameters, including concentration, motility and morphology (Moskovtsev *et al.*, 2009). In addition, up to 11% of normozoospermic, infertile men have significant DNA damage and 5% of infertile men with sperm parameters above the 50th percentile have significant DNA fragmentation (Zini *et al.*, 2002; Belloc *et al.*, 2014). While the American Society for Reproductive Medicine (ASRM) does not recommend the routine use of sperm DNA testing because of a concern that testing does not consistently predict treatment outcomes, there are data associating sperm DNA fragmentation with low potential for natural fertility, prolonged time to pregnancy, lower fertilization rates, impaired embryo progression and quality, decreased pregnancy rates in IVF/ ICSI cycles, and increased pregnancy loss after ART (Evenson *et al.*, 1999; Spano *et al.*, 2000; Robinson *et al.*, 2008; Simon *et al.*, 2014). These data are questioned on the basis of small study groups with inappropriate controls, and lack of validation of testing thresholds (Practice Committee of the American Society for Reproductive Medicine, 2013).

The etiology of sperm DNA fragmentation is likely multifactorial. While some data have supported a role for heat exposure (Paul *et al.*, 2008), others have implicated semen processing methods (Zini *et al.*, 2000, Nabi *et al.*, 2014). Nevertheless, oxidative stress is thought to be a common denominator in sperm DNA damage (Barratt *et al.*, 2010; Practice Committee of the American Society for Reproductive Medicine, 2013). Repetitive washing of sperm by serial centrifugation is associated with the generation of reactive oxygen species (Zalata *et al.*, 1995). Standard semen processing by density-gradient centrifugation has also been associated with decreased DNA integrity (Zini *et al.*, 2000). However, others have demonstrated similar (Malvezzi *et al.*, 2014) or reduced (Wang *et al.*, 2014; Fariello *et al.*, 2009) DNA fragmentation after density-gradient centrifugation compared with unprocessed samples.

Microfluidics has emerged as a high-throughput, automated and sensitive platform for application in various areas including biological and chemical analysis, point-of-care testing, forensic analysis and medical diagnostics (Song *et al.*, 2009; Zhang *et al.*, 2011; Tasoglu et al., 2013a, 2013b; Knowlton *et al.*, 2015; Reece *et al.*, 2016; Azo *et al.*, 2016). In recent years, microfluidics has been increasingly incorporated into the field of cryobiology and ART. Several microfluidic devices have been explored to sort motile and morphologically normal sperm from a raw sample without centrifugation, thereby potentially avoiding oxidative stress and DNA damage (Cho *et al.* 2003; Schuster *et al.*, 2003; Zhang *et al.* 2011; Tasoglu et al., 2013a, 2013b; Asghar *et al.* 2014; Shirota *et al.*, 2016; Chinnasamy *et al.*, 2017). Historically, the complexity of using microfluidic devices and the reliance on laminar flow, requiring a pump or gravity-dependent structure, have been barriers to clinical implementation. Additional limitations have included a selection efficiency that is unsatisfactory for samples with low sperm counts, prolonged processing times precluding use in the ART clinic, and the inability to completely eliminate dead sperm and debris in the absence of a filtering step (Schuster *et al.*, 2003; Cho *et al.*, 2003; Shirota *et al.*, 2016).

We introduce a mechanism for sperm sorting involving a space-constrained microfluidic sorting chip that is rapid, flow and chemical-free, and involves just two pipetting steps. A biodesign for channel length, height and travel time maximizes the sorting capability and recovery efficiency for ICSI. We sought to determine if the space-constrained microfluidic sperm separation method improves the selection of sperm for use with ICSI, with lower DNA fragmentation over standard density-gradient centrifugation with a swim-up step in split semen samples.

Materials and Methods

This is a blinded, controlled laboratory study of differences in standard semen analysis parameters and the DNA fragmentation index (DFI) in split samples that were discarded after routine semen analysis. This study was deemed exempt from Institutional Review Board review or written informed consent as the samples utilized were not linked to personal identifiers.

Semen analysis and processing

Discarded fresh routine semen analysis samples (n = 70) were consecutively collected. Semen analysis was performed by manual count of unprocessed samples following liquefaction. Microscopic examination included assessment of concentration, progressive motility (expressed as a %), and forward progression (overall assessment of sperm speed and directionality). For each sample, an aliquot of unprocessed semen was set aside for DFI analysis and the remainder split for processing by density-gradient centrifugation with swim-up and sorting by a microfluidic chip. The processing of samples was performed by two individuals who coded all the slides.

Density-gradient centrifugation was performed with a single 2 ml layer of 90% ISolate media (Irvine Scientific, Santa Ana, CA, USA) in a 15 ml

conical tube. A sample (1 ml) of the specimen was layered on top of the ISolate and centrifuged at 149 *g* at room temperature for 10 min. After centrifugation, the pellet was removed and placed into a clean conical tube with 5 ml of Sperm Washing Medium (Irvine Scientific, Santa Ana, CA, USA) and subsequently centrifuged at 149 *g* for 5 min. The supernatant was removed and the pellet was resuspended in 4 ml of Sperm Washing Medium. The sample remained at room temperature to allow for a swim-up to occur for 30 min to 1 h and the top 300 ul was removed and used for analysis.

Microfluidic sperm sorting was performed with the FERTILE (Zymot) device (DxNow Inc., Gaithersburg, MD, USA), a single-use chip with an inlet sample chamber connected to an outlet collection chamber by a microfluidic channel. The dimensions of the microchannels between the inlet and outlet port hydrodynamically constrain the migration of compromised sperm while allowing motile sperm to progress to the outlet, as described previously (Zhang *et al.*, 2011; Tasoglu et al., 2013a, 2013b). The microfluidic chip was loaded with Sperm Washing Medium prior to the addition of semen. A total of 50 µl of semen was processed. The chip was subsequently incubated for 30 min at 37°C. Processed sample was collected from the outlet of the chip for assessment of DNA fragmentation.

Sperm DNA fragmentation

DNA fragmentation was assessed using the SpermFunc® Sperm DNA Fragmentation test kit utilizing the sperm chromatin dispersion (SCD) method (BRED Life Science, Shenzhen City GD, China). For this assay, intact, unfixed spermatozoa are immersed in an inert agarose microgel on a slide. An initial acid denaturation step is followed by application of a lysing solution to remove nuclear proteins. The slides are stained with Wright's staining solution (Merck, Darmstadt, Germany) and visualized under a bright field microscope. In the absence of DNA breakage, nucleoids with large halos of spreading DNA loops are seen. Nucleoids from spermatozoa with fragmented DNA, however, do not show a dispersion halo (Fernández *et al.*, 2003). The SCD test has been correlated with other sperm DNA fragmentation tests to various degrees (Ribas-Maynou *et al.*, 2013; Evenson, 2016), with one report demonstrating an *r* of 0.93–0.94 in comparison with the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labeling (TUNEL) assay and 0.87–0.99 for sperm chromatin structure analysis (Chohan *et al.*, 2006). The SCD test was selected for this analysis owing to ease of use of a commercially available kit and cost.

Interpretation of DNA fragmentation by the SCD method on stained slides was performed by personnel who were blinded to the method of processing. The DFI was defined as number of cells with fragmented DNA divided by the number of total cells on a slide. Up to 300 cells were counted per slide. On slides with fewer total cells, 300 high power fields (×40 magnification) were examined. All slides were read by two individuals, each blinded to the method of processing and to the other's results.

Statistics

Summary descriptive statistics, including semen analysis parameters, were calculated with median values and interquartile ranges (IQR) for variables with skewed distributions. Samples were divided into DFI quartiles based on the DFI in the unprocessed sample. Linear regression models were built to investigate any association between DFI and sperm concentration and progressive motility in unprocessed samples. All DFI values underwent angular (arcsine) transformation prior to statistical testing. Statistical analysis was performed by Wilcoxon Signed-Rank test of paired samples due to the non-parametric distribution of outcomes of interest. Statistical analyses were performed using STATA version 14 (Stata Corp., College Station, TX, USA). A value of $P < 0.05$ was considered significant.

Results

Semen analysis parameters by the method of processing are displayed in Table I. The concentration of sperm was decreased after the sperm cells were processed by the microfluidic chip compared to centrifugation. However, after microfluidic processing the progressive motility of the sample was 100% compared with a median of 91% (IQR: 86–95) after density-gradient centrifugation with swim-up ($P < 0.0001$). Sperm concentration and progressive motility by quartile of DFI in the unprocessed sample are depicted in Table II. Quartile of DFI in the unprocessed sample was predictive of progressive motility (coefficient −3.1, $P < 0.01$) but not concentration. The distributions of DFI in unprocessed and processed samples are depicted in Fig. 1. Staining was insufficient to allow for assessment of DFI in 21 of 210 samples. Samples processed by the microfluidic chip had significantly lower DFI compared to paired samples that were unprocessed or processed by density-gradient centrifugation. The median DFI for chip specimens was 0% (IQR: 0–2.4) while those processed by density-gradient centrifugation with swim-up had a median DFI of 6% (IQR: 3–11.5). Unprocessed samples yielding the highest DFI quartile (DFI range: 31–40%) had a median DFI of 15% (IQR: 11–19%) after density-gradient centrifugation and DFI 0% (IQR: 0–1.9%) after processing with the microfluidic chip ($P = 0.02$). Representative images of a sample processed by centrifuge and chip are displayed as Fig. 2a and b.

Table I Semen analysis parameters by method of processing ($n = 70$).

	Concentration[a]	Progressive motility (%)[a]
Unprocessed ($n = 70$)	82 (61–133) M/ml[b,c]	54 (47–61)[b,c]
Density-gradient centrifuge ($n = 70$)	8 (6–11) M/ml[c]	91 (86–95)[c]
Microfluidic chip ($n = 70$)	10 (1–20)/hpf	100 (100–100)

[a]Median (interquartile range)
Wilcoxon Signed-Rank testing:
[b]$P < 0.0001$ versus density-gradient centrifuge.
[c]$P < 0.0001$ versus microfluidic chip.
hpf, High-powered field.

Table II Semen analysis parameters by quartile of DNA fragmentation index in unprocessed samples.

DFI quartile[a,b], DFI range (%)	Concentration (M/ml)[b]	Progressive motility (%)[b,c]
Quartile 1 ($n = 20$), 11.5 (8.5–14)	106 (81–173)	58 (52–66)
Quartile 2 ($n = 18$), 17.5 (16–20)	74 (58–125)	55 (48–61)
Quartile 3 ($n = 14$), 24.5 (23.0–27.0)	102 (65–148)	51 (37–56)
Quartile 4 ($n = 17$), 37 (33–40)	62 (50–78)	51 (44–58)

[a]From unprocessed sample.
[b]Median (interquartile ranges).
[c]$P < 0.01$ (linear regression of DFI quartile on motility).
DFI, DNA fragmentation index.

Discussion

We demonstrate that microfluidic sorting of unprocessed semen allows for the selection of clinically usable, highly motile sperm with nearly undetectable levels of DNA fragmentation. Standard processing by density-gradient centrifugation with swim-up did not increase DNA fragmentation, yet paired samples processed with the microfluidic chip demonstrated a significantly lower DFI for the latter. Our results are consistent with those of a prior report which demonstrated increased efficiency of microfluidic-based sperm selection, with higher motility and lower DFI following microfluidic sorting when compared to centrifugation and swim-up methods in a population of healthy volunteers (Shirota *et al.*, 2016). We provide an added comparison to the DFI of the unprocessed sample in an infertile population. Furthermore, the proposed microfluidic technology offers a simpler, flow-free approach to sort sperm requiring no peripheral equipment or filtration step, while minimizing hands-on time.

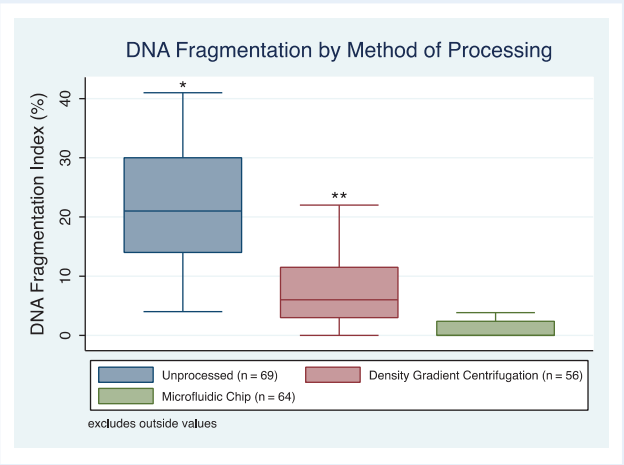

Figure 1 DNA fragmentation by method of processing human sperm. Box plots for each method of processing demonstrating median DNA fragmentation index (DFI) as horizontal value within shaded box, 25th and 75th percentiles as lower and upper bounds of shaded box, and whiskers demonstrating 1.5 times the upper or lower quartile. *$P < 0.0001$ versus chip and centrifuge **$P = 0.0029$ versus chip (Wilcoxon Signed-Rank Test for Paired Samples performed after arcsine transformation of DFI).

While some studies have suggested that centrifugation of semen samples is associated with the generation of increased reactive oxygen species and a higher DFI (Zini *et al.*, 2000), others have not (Malvezzi *et al.*, 2014; Wang *et al.*, 2014). These discrepant results may relate to differences in DNA fragmentation levels in whole semen in the population under study or in the methods for centrifugation. The swim-up method of sperm processing has been associated with improved DNA integrity (Zini *et al.*, 2000), but is also time-consuming, associated with prolonged exposure of motile sperm to semen, and not routinely performed clinically without an initial centrifugation step. While we did not perform an exclusive swim-up for comparison, we demonstrate that density-gradient centrifugation with a swim-up step resulted in the selection of sperm with decreased DFI as compared to unprocessed samples, while the use of the microfluidic sperm sorting device resulted in further reductions in DFI, consistent with others (Malvezzi *et al.*, 2014). It is notable that we did include a swim-up step following centrifugation to mimic processing for ART; thus, it is possible that had we evaluated the entire sample we would have seen a higher DFI post-centrifugation and, thus, a greater advantage to use of the microfluidic device. Additionally, the microfluidic chip is designed to undergo incubation at 37°C while the samples processed by density-gradient

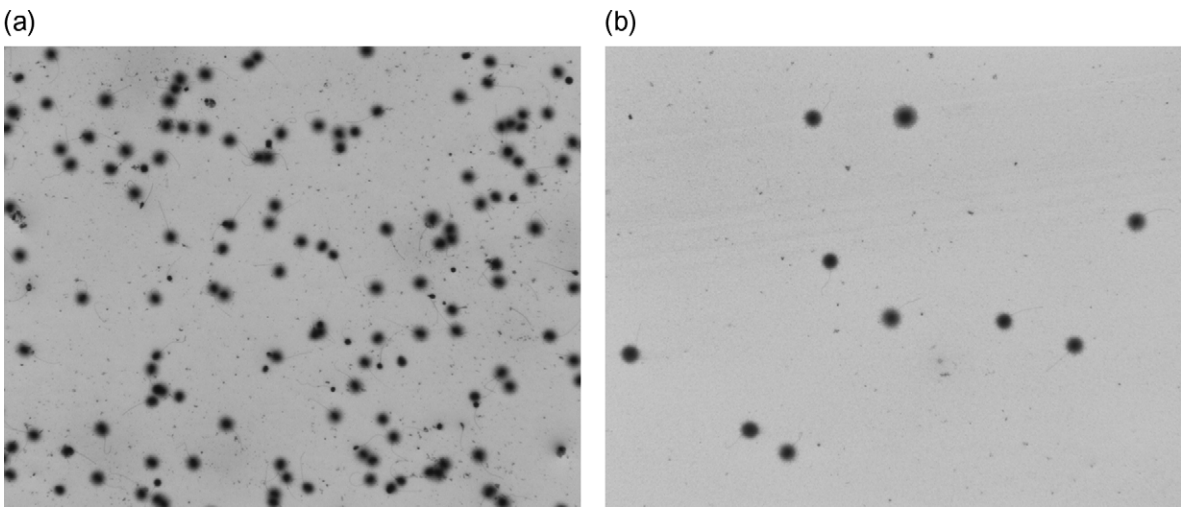

Figure 2 Representative images from the sperm chromatin dispersion assay of human samples processed by density gradient centrifugation or microfluidic chip. (**a**) Sperm chromatin dispersion (SCD) assay slide (×10 magnification) following processing with density-gradient centrifuge demonstrating 27% fragmented sperm cells containing little to no halo. The remaining cells demonstrate a large halo of dispersed DNA, which is consistent with the absence of fragmentation. (**b**) SCD assay slide (×10 magnification) following processing with microfluidic chip demonstrating 100% non-fragmented sperm cells containing large halos of dispersed DNA.

centrifugation underwent a similar duration of incubation for swim-up at room temperature. Prior reports have suggested that incubation at room temperature yields lower or equivalent DFI when compared with incubation at 37°C (Matsuura *et al.*, 2010; Repalle *et al.*, 2013). Thus, the difference in incubation temperatures between groups would only be expected to bias toward the null hypothesis, making our finding of improved DFI after processing with the chip compared with centrifuged samples more notable.

Microfluidic sperm processing offers an alternative to traditional sperm separation procedures wherein highly motile sperm are isolated from an unprocessed sample. While progressive motility and DFI are improved via processing by a microfluidic chip, sperm concentrations are consistently reduced, reflecting the highly selective nature of the device. Furthermore, a single microfluidic chip is not capable of processing an entire ejaculate and, therefore, will not isolate all motile sperm. Nevertheless, in our study, the processed samples uniformly had sufficient sperm for ICSI.

The clinical utility of the assessment of sperm DNA fragmentation remains in question because of inconsistent associations between DNA integrity and clinical outcomes. However, variable results may relate to small sample sizes, inappropriate or absent controls, and poorly validated thresholds for test results (Practice Committee of the American Society for Reproductive Medicine, 2013). Furthermore, retrospective reports of outcomes between patients with a high or low DFI may have failed to reveal a consistent clinical impact due to the unmatched nature of these comparisons, i.e. individuals with high or low DFI are likely to differ in other important ways that may have a greater association with clinical outcomes. Finally, assessment of DNA fragmentation is usually performed on samples not used clinically. Therefore, it is unclear whether a DFI assessment performed on a diagnostic sample is truly representative of the sperm selected for fertilization in a treatment cycle in which clinical outcomes are obtained.

Nevertheless, high levels of DNA fragmentation have been associated with poor outcomes in IVF, in particular, poor blastulation and increased rates of pregnancy loss (Larson-Cook *et al.*, 2003; Seli *et al.*, 2004; Zini *et al.*, 2008). As a result, antioxidants, and, for some, surgical sperm retrieval have been advocated for men with high levels of sperm DNA fragmentation (Bradley *et al.*, 2016; Esteves *et al.*, 2015). However, in our study, processing with a microfluidic sperm sorting device allows for a reduction in DFI from >30% in the highly fragmented unprocessed samples to near undetectable levels, without the need for medical or surgical intervention. There has not previously existed an opportunity to process sperm for clinical use to achieve an undetectable level of DNA fragmentation. Randomized studies are needed to determine if this selection affords a difference in clinical outcomes.

Study strengths, limitations and future directions

As this is a laboratory study using discarded, de-identified samples, no clinical data are available to describe the population under study. Nevertheless, given the inclusion of samples from patients undergoing evaluation for infertility rather than healthy volunteers or previously identified male factor cases, it is likely to represent the population being seen and treated in an infertility practice. Had we limited the study to those with known male factor infertility, we would likely have seen a greater impact of processing with microfluidic sperm sorting due to a higher baseline DFI (Belloc *et al.*, 2014) and an even greater potential for improvement with a highly selective device.

Additionally, while data have demonstrated associations between high DFI and poor outcomes with IVF/ICSI, there are limited data illustrating improvements in clinical outcomes with reduction in DFI. As this study utilized discarded, non-clinical samples we do not have clinical outcome data. For this reason, we have initiated a RCT of microfluidic sperm sorting versus standard sperm processing to examine differences in meaningful clinical outcomes between these interventions (NCT 030854233).

Conclusion

Microfluidic sorting of unprocessed semen allows for the selection of clinically usable highly motile sperm with nearly undetectable levels of DNA fragmentation.

Authors' roles

M.M.Q. was involved in the acquisition, analysis, and interpretation of data in addition to drafting and revising the article. L.Z., S.R. and K.O. were involved in the acquisition and analysis of data in addition to the revision of the article. M.I.C. provided contributions to conception and design of the article and its revision. U.D. and M.P.R. were involved in the conception and design of the article, interpretation of data and drafting/revision of article. All authors have given final approval of the version of the article to be submitted for consideration.

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None.

Conflict of interest

U.D., PhD is the co-founder and Scientific Advisor for DxNow Inc., LevitasBio Inc., and Koek Biotech. Mitchell Rosen, MD is a member of the Clinical Advisory Board for DxNow Inc.

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