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Research Article

Lectin-Functionalized Magnetic Iron Oxide Nanoparticles for Reproductive Improvement

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Abstract

Background: Semen ejaculates contain heterogeneous sperm populations that can jeopardize male fertility. Recent development of nanotechnology in physiological systems may have applications in reproductive biology. Here, we used magnetic nanoparticles as a novel strategy for sperm purification to improve semen fertility.

Methods: Boar semen was obtained in artificial insemination doses from a local boar stud. Doses were mixed with or without magnetic nanoparticles designed to target and deplete moribund and poor performing spermatozoa under an electromagnetic field. Sperm motility characteristics were assessed prior to insemination of open gilts with control (n=3 gilts) and nanopurified (n=4 gilts) semen. Pregnancies were verified 30 days post-insemination. Litter sizes and post-natal development of piglets were respectively evaluated at parturition and weekly until weaning.

Results: Nanopurification significantly improved sperm motility. Two gilts in the control group were confirmed non-pregnant, but the remainder maintained pregnancies through to parturition (33% vs. 100%, control and nanopurified groups, respectively). At parturition, the number of piglets born to the control gilt was not significantly different from the average of the nanopurified group (17 ± 0.0 vs. 15 ± 2, respectively; P>0.05); however, in the latter group 78% of piglets remained alive compared to 76% of the control. Birth weight of control piglets was lower (1.18 ± 0.22 kg) than those in the nanopurified group (1.41 ± 0.14 kg). Both groups of piglets showed linear and parallel growth rates with respective weight gains of 4.4x and 4.1x from birth to weaning. Interestingly, piglets produced in the nanopurified group comprised of 55% males compared to 38% in the control group.

Conclusions: Magnetic nanoparticles used in this preliminary study exhibited no toxic effects on sperm fertilization capacity and piglet viability. Beneficial effects may be seen in semen fertility, with possible use for gender selection. Further investigations on a large scale are needed to confirm the current findings, with potential application in clinical practice.

Keywords: Assisted reproduction; Fertility; Nanoparticles; Pig; Spermatozoa; Sex ratio; Swine

Introduction

The consistent production of high quality spermatozoa is crucial for male fertility. Numerous factors associated with the male (i.e., age, health, genetic line, nutrition status [1-3]) and the environment (i.e., seasonal changes of temperatures, cryopreservation [1,4,5]) are known to affect semen quality. Most importantly, semen normally contains a heterogeneous population of spermatozoa with attributes that have a significant impact on male fertility potential [6,7]. For example, semen collected from normal fertile males usually results in significantly low proportions of abnormal spermatozoa and higher proportions of motile spermatozoa, with better viability (i.e., intact acrosome, plasma and mitochondrial membranes, low DNA fragmentation index) than those collected from low fertile males [8,9].

In many agricultural and clinical laboratories, routine practices of semen quality analyses for artificial insemination purpose are often limited to the evaluation of sperm concentration, motility, and morphology due to the rapidity of the tests. Yet, these parameters are relatively poor predictors of semen fertility [1,10]. In contrast, the aforementioned viability factors are strong predictors of semen fertility due to their tremendous impacts on sperm progression within the female genital tract and interaction with the oocyte at the site of fertilization [9,11,12]; however, their evaluation can be laborious and results are often only available after the preparation of insemination doses. Although current available tests can be effective at quantifying the proportions of viable spermatozoa within semen ejaculates or doses for artificial insemination [13], the removal of damaged spermatozoa would be of great benefit. This procedure may contribute to the elimination of the needless competition between viable and non-viable spermatozoa, leading to a higher number of viable spermatozoa reaching the fertilization site for improved fertility.

To this end, the recent progress in nanotechnology offers an excellent opportunity for the selective removal of damaged or defective spermatozoa. Nanotechnology refers to the technology dealing with particles synthesized at the nano-scale level (1 to 100 nanometers). These nanoparticles can be tailored to different sizes and composition, and their biocompatibility with biological fluids makes them an excellent device for cell targeting and non-targeting interactions for fluorescence or magnetism purposes [14]. Despite their reported toxicity in certain experimental conditions [15,16], nanoparticles have been successfully used in various aspects of biomedicine, including cancer treatment [16]

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and reproduction [17-19].

For cell targeting purpose, lectin and carbohydrate receptor systems found on sperm surface plasma membranes can serve as great candidates for labeling. Lectins are glycoproteins capable of inducing sperm agglutination through their binding to plasma membrane carbohydrates. Yet, numerous studies have used exogenous lectins to investigate the dynamics of carbohydrates on normal, capacitated, and acrosome reacted spermatozoa of fertile and subfertile males, including boars [20-23]. A recent study that used lectin-coated magnetic nanoparticles to remove abnormal spermatozoa from bovine semen ejaculates, resulted in improved sperm fertility after artificial insemination [19].

Hence, the objective of this study was to test the efficacy of the novel magnetic nanoparticles designed specifically to target moribund spermatozoa in boar semen prepared for artificial insemination. The observed outcomes included the evaluation of the nanoparticle treatment on semen fertilizing potential, prolificacy of inseminated gilts and post-natal development of piglets.

Materials and Methods

Animal care and use were performed according to protocols approved by the Institutional Animal Care and Use Committee of Mississippi State University.

Magnetic nanoparticles

Iron oxide (Fe2O3) nanoparticles were coated with lectins (PNA/PSA; Sigma Aldrich, St Louis, MO, USA) to selectively bind to the glycan molecules exposed on the inner membrane of acrosome-reacted spermatozoa. Functionalized magnetic nanoparticles were synthesized (Clemente Associates; Madison, CT, USA) and provided for sperm labeling in a stock of 14.1 mg/ml of 10 mM phosphate buffer without sodium azide. The stock was stored at 4°C until use.

Sperm labeling, nanopurification, and motility analysis

Boar semen was harvested and aliquots prepared in bags of artificial insemination doses (approximately 3×10^9 spermatozoa in 80 ml Beltsville Thawing Solution) at a local boar stud (Prestage Farms, West Point, MS, USA). Each bag of semen was mixed with 90.2 mg nanoparticles and placed under a gentle rotation for 30 minutes at 37°C. Afterwards, mixtures were placed against an external 7 magnetic field consisting of magnets fixed in polycarbonate. Free and sperm-bound magnetic nanoparticles were pulled down to the wall of the bag and nanoparticle-free spermatozoa were collected into different 50-mL Falcon centrifuge tubes. The process was repeated 3-4 times until the maximal removal of nanoparticles was achieved (Figures 1 and 2). Aliquots (500 µl) of control (non-purified) and nanopurified semen were incubated for 10 minutes at 37°C before analyses of sperm motility characteristics using a Computer Assisted-Sperm Analyzer (Hamilton-Thorne; Beverly, MA, USA). Both control and nanopurified semen were then immediately transported (at 20 ± 1°C) to the farm for insemination.

Estrus detection and artificial insemination

Seven maternal-line open gilts approximately 10 months of age were maintained at the Physiology Unit, H. H. Leveck Animal Research Center, Mississippi Agricultural and Forestry Experiment Station, Mississippi State University (MSU-MAFES). Estrus was determined using the standard "Standing Reflex" method. Gilts detected in estrus were artificially inseminated 2 to 3 times within the next 24 hours, starting from 6 hours post-detection. Three gilts were inseminated with control semen and four with nanopurified semen.

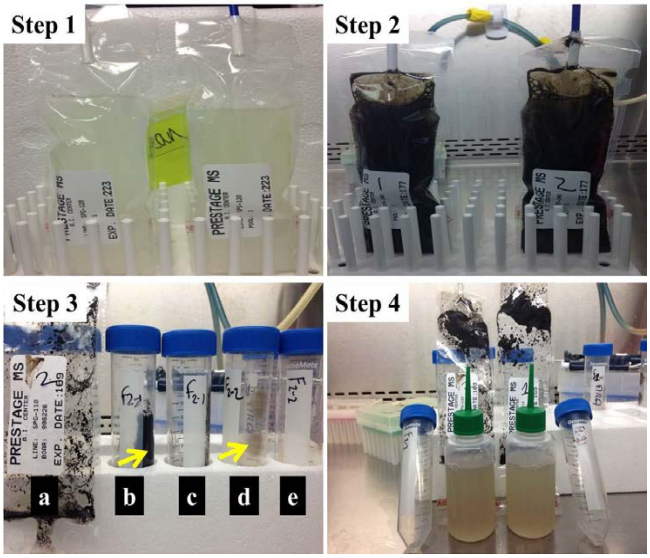

Figure 1: Labeling of boar spermatozoa and nanopurification using magnetic nanoparticles
Insemination doses of boar semen (**Step 1**) were mixed with magnetic nanoparticles (**Step 2**). After labeling, mixtures were exposed to an external magnetic field to trap all magnetic nanoparticles (free and those bound to defective spermatozoa). Nanoparticle-free spermatozoa were then poured off into centrifuge tubes, which were subsequently placed onto the magnetic field for a new purification procedure that was repeated 3-4 times (a, b, c, d, and e in **Step 3**) for the maximal removal of nanoparticles (see arrows). Nanopurified semen samples were then transferred into insemination bottles (**Step 4**).

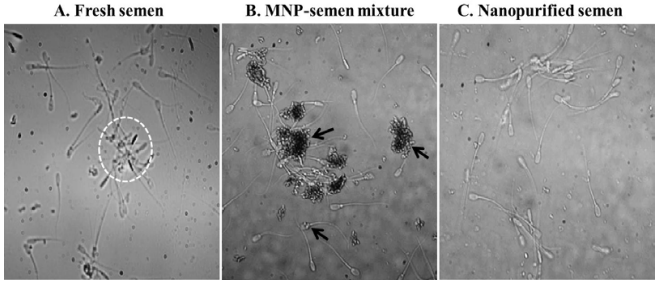

Figure 2: Sperm nanopurification
Micrographs represent fresh semen (**A**) of boars, mixed with magnetic iron oxide nanoparticles or MNP (**B**), and MNP-free spermatozoa following (nano) purification (**C**). The circle shows aggregates of spermatozoa that will be removed through the formation of MNP-sperm complexes (arrows) after purification within a magnetic field. Nanopurified semen was enriched with individual and highly motile spermatozoa.

Female fertility and piglet measurements

Pregnancy confirmation was performed at 30 days post-insemination using an ultrasonic detector and results were used to calculate the pregnancy rate for each group of pigs inseminated with control (n=3) or nanopurified (n=4) semen. All animals were monitored throughout the pregnancy, and only the confirmed pregnant gilts were transferred into farrowing stalls a few days prior to predicted delivery. Then, the linear body measurements of the (heart) girth and length were used to estimate the weight of gilts according to Groesbeck et al. [24]. The length was measured, dorsally, between the base of the ear to the base of the tail and the heart girth corresponded to the gilt circumference just behind the forelegs.

At 12 hour post-partum, piglets were removed from the dam for measurements. The litter size of each gilt was recorded, the gender of piglets noted, and ears were notched for individual piglet identification. In addition, piglets were weighed and the crown-to-rump length (CRL) was measured from the crown of the head to the base of the tail. Data were collected and assessed weekly during the nursing period for the detection of any negative effects of the semen nanopurification procedure.

Statistical analysis

All data were analyzed and compared using the IBM SPSS Statistic package (version 22). The nanopurification effects on sperm motility characteristics, litter sizes, and post-natal measurements (weight and CRL) were performed with the Student's t-Test and one-way ANOVA when necessary. The Chi square test was used to evaluate the semen effect on the sex ratio and the repeated measurements of ANOVA was used for the overtime analyses of weight and CRL of neonate pigs. Values of P less or equal to 0.05 were fixed as the threshold of significance. Data are expressed as mean ± SEM, unless otherwise indicated.

Results and Discussion

Magnetic nanoparticles

The selection of lectins for coating the nanoparticles was based on their essential role during cell recognition [25]. Numerous lectins have been described on the sperm surface of various species and their ability to bind carbohydrates present on oviductal epithelia surface membrane and zona pellucida of the oocyte allows species-specific interactions [23,26,27]. The oviduct uses these interactions to select high performing spermatozoa with features such as superior morphology and intact acrosome. Furthermore, capacitated and acrosome reacted spermatozoa possess specific carbohydrates or glycans that interact with lectins of neighboring spermatozoa to induce head-to-head agglutinations [19-23,25-28], leading to lesser spermatozoa in the vicinity of the oocytes. Thus, the technical approach proposed in this study is innovative in swine reproduction as it uses magnetic nanoparticles coated with PNA lectin to selectively target acrosome reacted spermatozoa, followed by their removal from semen doses for artificial insemination. The proposed approach has the potential to provide a high number of viable spermatozoa in semen doses for successful inseminations.

Effect of nanopurification on sperm motility

First, we evaluated the impact of magnetic nanoparticles on the motility of nanopurified spermatozoa. All samples were analyzed at least twice and data are summarized in Figures 3 and 4. The nanopurification procedure using the designed magnetic nanoparticles resulted in the selection of spermatozoa with higher motility (P<0.05; Student's t-Test). The proportion of motile, including spermatozoa moving straightforward (progressive) and fast (rapid, >30 µm/sec) were all increased compared to the control group (Figure 3). Moreover, no negative effects of the nanopurification process were found on other motility parameters such as the curvilinear (VCL), straight-line (VSL) and average path (VAP) velocities, the amplitude of lateral head displacement (ALH), and the beat cross frequency (BCF).

Because the magnetic nanoparticles used were designed (lectin-coated) to interact with the glycan molecules only available on the inner membrane of acrosome-reacted spermatozoa, the current findings suggest that the removal of defective spermatozoa from the semen allowed the enrichment of insemination doses with high motile (motile and progressive) and viable (intact acrosome) spermatozoa. These motility parameters have been associated with *in vitro* and *in vivo* fertility of boar semen [29] and here, results with nanopurified semen are in agreement with the recent findings using fresh and frozen-thawed bovine semen [19].

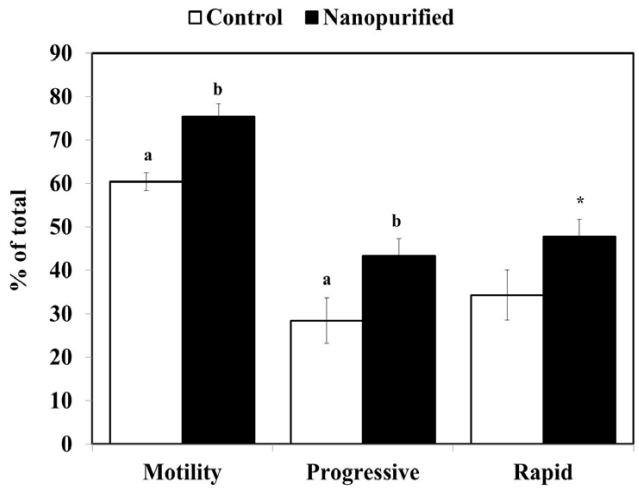

Figure 3: Effect of magnetic nanoparticle purification on porcine sperm motility.
Data are mean ± SEM. Different letters (a,b) indicate significant difference between groups for each analyzed parameter (P<0.05; Student's t-Test). The asterisk indicates a tendency toward a significant difference between the two groups (P=0.07; Student's t-Test).

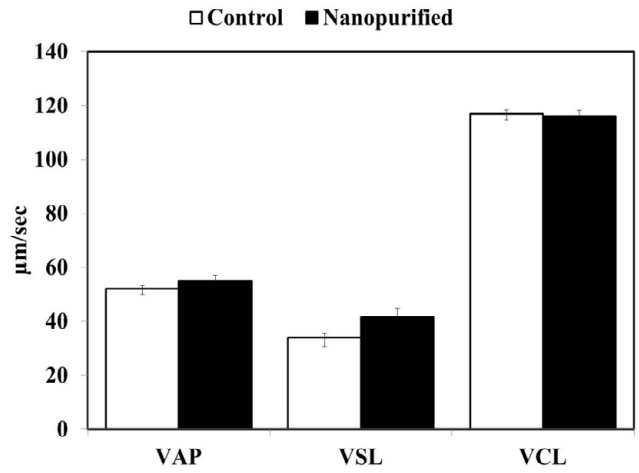

Figure 4: Effect of magnetic nanoparticle purification on porcine sperm velocity
Data are mean ± SEM. No significant differences were found between the two groups, for each VAP (Average Path Velocity), VSL (Straight-Line Velocity), and VCL (Curvilinear Velocity) parameters (P>0.05; Student's t-Test).

Furthermore, it is important to mention that the nanopurification procedure increased the motility parameters defining the progressiveness of spermatozoa [VSL: 41.5 ± 2.3 µm/sec vs. 34 ± 1.6 µm/sec; straightness (VSL/VAP)*100: 73 ± 1.8% vs. 64 ± 1.9%; linearity (VSL/VCL)*100: 38 ± 1.5% vs. 31 ± 1.2%], while those (VCL, ALH, and BCF) indicative of the vigor of spermatozoa were not affected. A recent study in pigs found that sperm progressiveness, VSL, linearity, and acrosome reaction parameters are all together great predictors for successful insemination and breeding performance in pigs [9]. The current improved motility characteristics with the reported viability of nanopurified spermatozoa in bovine [19] allow for an expectation of a fertility enhancement of nanopurified semen in pigs.

Semen group	Total number of gilts		Number of piglets born per litter		Number of male
	Inseminated	Pregnant (%)[1]	Total	Alive at 24-h (%)[2]	Piglets alive** (%)[3]
Control	3*	1 (33)	17.0 ± 0.0	13.0 ± 0.0 (76)	5.0 ± 0.0 (38)
Nanopurified	4	4 (100)	14.8 ± 0.9	11.5 ± 0.9 (78)	6.5 ± 0.9 (56)
P value		ND	0.1	0.22	0.39

Proportions are ratios between the numbers of [1]"Pregnant" over "Inseminated" gilts, [2]"Alive at 24-h" over "Total" born per litter, and [3]male piglets over the corresponding "Alive at 24-h".

*Two gilts were confirmed positive by the pregnancy test at 30 days post-insemination, but only one maintained full term pregnancy. **Data were collected after the nursing period.

The Student's t and Chi square tests were used to determine the effect of the semen treatment on the litter sizes and the gender selection, respectively. The effect on the pregnancy rate was not evaluated due to the low number of pigs (ND= Not determined). No significant differences were found between groups (P>0.05). Data are expressed as mean ± SEM.

Table 1: Evaluation of sperm nanopurification on pig fertility.

Effect of sperm nanopurification on sow fertility

Seven open gilts were fed and maintained under the same conditions throughout the experiment. As shown in Table 1, inseminations with control semen led to two pregnancies out of three gilts at 30 days post-insemination, but only one maintained a full term gestation (33% pregnancy rate). In contrast, gilts bred with nanopurified semen (n=4) were all pregnant and maintained pregnancy until successful farrowing (100% pregnancy rate). Prior to farrowing, the heart girths and lengths of gilts inseminated with control semen were respectively measured at 1.57 ± 0.0 m and 1.52 ± 0.0 m versus 1.51 ± 0.1 m and 1.57 ± 0.04 m for those inseminated with nanopurified semen. These data were then used to estimate the gilt weights which appeared comparable between the control (260 ± 0 kg) and the nanopurified (249 ± 32 kg) groups (means ± SD; P>0.05). This method has been reported to be highly accurate for body weight estimations [24,30]. The observed results on dams lead us to consider that any potential differences at the piglet level would be attributable to the semen quality, unless there are undetectable issues associated with the bred gilts. Thus, with regard to the low pregnancy rate of the control group, one may speculate that the findings are indicative of a possible beneficial effect of semen nanopurification on the pregnancy rate. However, a strong evidence of such effect remains to be confirmed through a large scale study that includes higher number of animals.

Data summarized in Table 1 also indicate the number of piglets born to gilts inseminated with control (17.0 ± 0.0) and nanopurified (14.8 ± 0.9) semen. Only 13 ± 0 and 11.5 ± 1 piglets were alive at 24-h post-natal, corresponding to 76% for the control and 78% for the nanopurified group. The average litter size of the nanopurified group was not significantly different from the control sow (P>0.05). Interestingly, the nanopurified group revealed a higher proportion of viable male piglets (56%) in comparison to the control group (38%). The findings suggest that the nanopurification of boar spermatozoa prior to insemination does not affect their fertilizing capacity and fertility potential. Further studies involving a larger number of gilts in each group are needed to determine whether nanopurification of semen leads to a possible gender shift in favor of males.

Effect of sperm nanopurification on post-natal development of piglets

Piglets born to the gilts inseminated with control semen were smaller than their counterparts in the nanopurified group, at 12-h (1.18 ± 0.1 kg vs. 1.31 ± 0.0 kg) and 24-h (1.28 ± 0.1 kg vs. 1.4 ± 0.1 kg) post-natal (P>0.05). Piglets in both groups grew linearly during the nursing period, with control-derived piglets remaining numerically smaller than those obtained from nanopurified semen (Figure 5; P>0.05). Similarly, no significant differences were found between the two groups when considering the piglets' CRL during the nursing (or pre-weaning) period (Figure 6; P>0.05). The findings clearly indicate that the designed magnetic nanoparticles had no negative or toxic effects on the post-natal development of piglets, at least, during the nursing period.

Conclusion

Altogether, the present study proposes the use of magnetic nanoparticles for specific targeting and depletion of moribund and poor performing spermatozoa from semen doses through an external magnetic field. This novel strategy has potential application as a viable and non-invasive alternative tool for the enrichment of semen doses

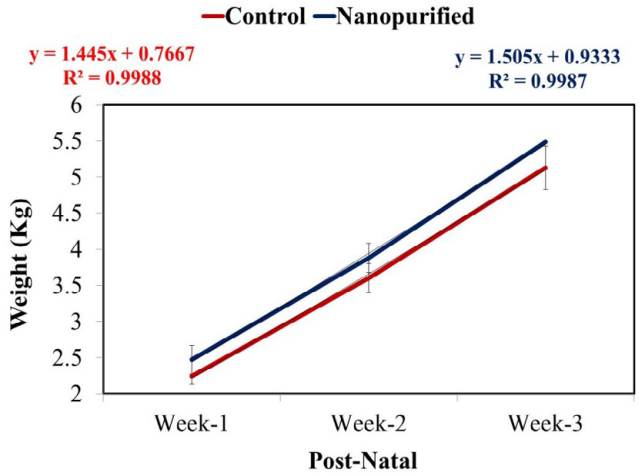

Figure 5: Evaluation of the magnetic sperm nanopurification on the weight of neonate pigs
Data are mean ± SEM. No significant differences were found between the two groups at each time-point (P>0.05; ANOVA 1). However, significant differences were found within groups over the time (P<0.05; repeated measurements of ANOVA).

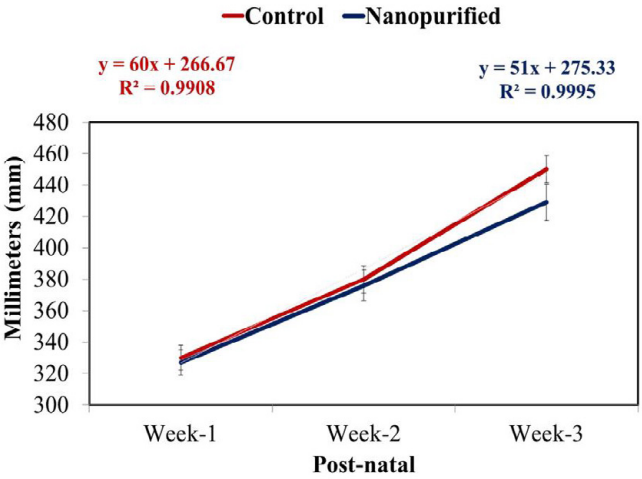

Figure 6: Evaluation of the magnetic sperm nanopurification on the crown-to-rump length of neonate pigs.
Data are mean ± SEM. No significant differences were found between the two groups at each time-point (P>0.05; ANOVA 1). However, significant differences were found within groups over the time (P<0.05; repeated measurements of ANOVA).

with high quality spermatozoa to enhance fertility performance. We further believe that this technology has a promising future in both human and livestock, although additional investigations are still needed to confirm and expand the present findings. These investigations should focus on the detection of possible nanoparticles-induced effects that may impair normal embryogenesis and post-natal development.

Authors' Contributions

JMF conceived and designed the study, performed most of the experiments, drafted and wrote the manuscript; SFL and MAC contributed to the experimental design, participated to the daily swine management (feeding, breeding, pregnancy and farrow, and nursery of piglets), and help drafting the manuscript; HC designed and prepared the magnetic nanoparticles, contributed to the experimental design, and assisted on data interpretation; PLR and STW participated to the experimental design, provided guidance, and help interpreting results. All authors have discussed the results and reviewed the manuscript. All authors read and approved the final manuscript.

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