NBMA Promotes Spermatogenesis by Mediating Oct4 Pathway

Jinfei Yang*, Dengfeng Lin*, Weiwei Yao, Damin Yun, Liwei Zhou, Sheng Gao, and Fei Sun*
Table of Contents

1. Reaction Optimization .............................................................S2
2. The Effect of NBMA on Spermatogenesis ..............................S3
3. mRNA-seq Analysis .................................................................S3
4. qPCR Analysis and Western Blot .............................................S4
5. ELISA Analysis ........................................................................S6
6. NMR Spectra ...........................................................................S7
1. Reaction Optimization

![Reaction Scheme]

| entry | catalyst  | solvent     | T (°C) | yield 3a (%)[^b] |
|-------|-----------|-------------|--------|-------------------|
| 1     | MnBr₂     | DMF         | 80     | 0                 |
| 2     | MnBr₂     | DMSO        | 80     | 0                 |
| 3     | MnBr₂     | CH₂CN       | 80     | 0                 |
| 4     | MnBr₂     | Dioxane     | 80     | 10                |
| 5     | MnBr₂     | THF         | 80     | 8                 |
| 6     | MnBr₂     | DCE         | 80     | 9                 |
| 7     | MnBr₂     | p-Xylene    | 80     | 29                |
| 8     | MnBr₂     | Mesitylene  | 80     | 32                |
| 9     | MnBr₂     | Toluene     | 80     | 41                |
| 10    | MnBr₂     | Toluene     | 60     | 30                |
| 11    | MnBr₂     | Toluene     | 90     | 58                |
| 12    | MnBr₂     | Toluene     | 100    | 58                |
| 13[^c] | MnBr₂   | Toluene     | 90     | 63                |
| 14[^d] | None     | Toluene     | 90     | 0                 |

Table S1 Effects of temperature, and solvent. N-benzyl-4-methoxyaniline (0.2 mmol), 1-ethynyl-4-(trifluoromethyl)benzene (0.4 mmol), MnBr₂ (0.08 mmol), solvent (2.0 mL), at 90 °C for 24 h. [a] The reactions were carried out in sealed tubes. [b] Yields were determined by GC analysis. [c] 72 h. [d] Without MnBr₂.

**NBMA**, White solid (48 mg, 63% yield).^1^H NMR (400 MHz, CDCl₃): δ 7.57 (d, J = 8.0 Hz, 2H), 7.46 (d, J = 8.0 Hz, 2H), 7.24 – 7.20 (m, 3H), 7.03 – 7.00 (m, 2H), 6.84 (dd, J = 8.8, 2.3 Hz, 1H), 6.75 (d, J = 2.8 Hz, 1H), 6.63 (d, J = 8.8 Hz, 1H), 5.88 (d, J = 0.8 Hz, 1H), 5.50 (d, J = 1.2 Hz, 1H), 4.18 (s, 2H), 3.77 (s, 3H); ^1^C NMR (100 MHz, CDCl₃): δ 151.6, 146.0, 143.2, 139.4 (d, J_C-F = 7.7 Hz), 130.1 (q, J_C-F = 32.2 Hz), 128.5, 128.2, 127.8, 127.1, 127.1, 126.9, 125.5 (q, J_C-F = 3.8 Hz), 122.8, 118.6, 116.8, 114.4, 112.3, 55.8, 48.9.
2. The Effect of NBMA on Spermatogenesis

Busulfan destroys DNA structure, prevents proliferation and differentiation of spermatogonia stem cells (SSCs), causing the spermatogenesis disrupted. Four weeks after the busulfan injection, the testicular size, weight, and sperm concentration were all remarkably reduced compared with the control group. The mice received the NBMA showed significant recovery in their testicular weights and spermatozoa counts. Four weeks after the gavage of the NBMA, busulfan treated mice, received 10mg/kg N MBA and 30mg/kg NBMA, were close to the control group at testis weights and sperm counts level.

We observed the phenotype of the seminiferous tubules by HE staining. As shown in the figure 3, at week 2 after NBMA gavage, we found that there was obvious vacuolation in the seminiferous tubules in group BD (Busulfan+DMSO), the number of germ cells was significantly reduced, all spermatids and most of the spermatocyte were disappeared, 91% of tubules have only spermatogonia left in the basement membrane. By contrast, atrophic seminiferous tubules of the NBMA administration Group were distinct reduced. In group BD+0.1mg/kg, only 24.5% tubules had spermatogonia alone, 72% tubules had spermatocytes, even 3.5% tubules had recovered with spermatids. What’s more, with the increment of the NBMA dose, 21% of tubules had spermatids in Group BD+30mg/kg. At week 4, only 9% of tubules had spermatids, while the group BD+1mg/kg and the group BD+30mg/kg almost had normal spermatogenesis, with about 90% tubules having spermatids. In a word, NBMA promote the recovery of germ cells in the Busulfan mouse model.

3. mRNA-seq Analysis

To explore how NBMA promotes spermatogenesis in a busulfan-induced testis toxicity mouse model and investigate the mechanism, we randomly selected two RNA samples of testis tissues from the BD group and BD+30mg/kg group at week two after NBMA gavage, respectively for mRNA sequence analysis. The data summary is
shown in the Figure 5. Compared to the BD group, there were 333 genes up-regulated and 1268 genes down-regulated for 4 times in the BD+30mg/kg group. The upregulated genes were analyzed using GO analysis. The genes were enriched in spermatogenesis and reproduction-related functional pathways, which indicates that NBMA may play an essential role in spermatogenesis.

Furthermore, we selected spermatogonia development-related genes from spermatogenesis, male gamete generation, spermatid differentiation, spermatid development, and reproduction, these five functional pathways. Then, we found the upregulated expression of Dazl, which are essential for spermatogonia proliferation and differentiation, is remarkable up regulation. The data suggested NBMA may promote spermatogenesis by strengthening the expression of Dazl.

4. qPCR Analysis and Western Blot

To further verify our result of RNA-seq, the mRNA and protein expression of Dazl and its target genes was detected by qPCR analysis and western blot. The mRNA levels of Dazl, Ddx4, Lin28a, Foxo1, Id4, Sall4, Zbtb16, Taf4b, Sohlh1, Sohlh2, Sycp1, Sycp3 were significantly decreased in the BD group, while these genes were highly expressed after NBMA gavage and recovered to approximately normal levels. The protein levels of germ cell-specific genes, Dazl, Ddx4, and Sycp3, were obviously upregulated after given NBMA of 30mg/kg verse the BD group. The data suggests that NBMA may promotes the development of spermatogonia to spermatocytes and on to spermatids by increasing the expression of Dazl and its target genes.
Table S2. Primers used for qPCR

| Genes | Primer sequence | Product size(bp) |
|-------|----------------|-----------------|
| Pax5  | F:AACTTGCCCATCAAGGTGTC R:CTGATCTCCAGGCAAAACT | 217 |
| Dazl  | F:AAGGCAAAATCATGGCCAACAC R:TCTGCACTCCAGCTTATT | 184 |
| Ddx4  | F:TGGCAGAGGATTTCTTTTT R:CGCTGTATTCAACGTTGGCT | 226 |
| Lin28a| F:CAGAAGCGAAGATCCAAAGG R:CAGGCTTTCCCTGAGAACTG | 178 |
| Sohlh1| F:CATCTGCTGTTGTCTCGGTA R:GCTGGAAGACTCTGCGTCAC | 161 |
| Sohlh2| F:TCTCAGCCACATCAGCACAGG R:GGGGACGCGAGTCTTATACA | 200 |
| Sall4 | F:GCCCTCTCAACTGTCTCCTC T:GAGACTCACCCTGCTTTGCT R:AGAATGCTGTCACCCTGCTT | 150 |
| Foxo1 | R:GGAGGCTGTTTTCTCGACTG | 211 |
| Id4   | F:GCCCTCTCAACTGTCTCCTCTG R:GCTGGAAGACTCTGCGTCAC | 152 |
| Zbtb16| F:AGAATGCTGTCACCCTGCTT R:AACGGTTCCTGGACAGTTTG | 173 |
| Taf4b | R:CCCAACACACAGACAGAGAAGA | 228 |
| Sycp1 | R:TCTCAGGCTGGAAGGGACACT F:AGGTTGAGAAAGCACAAGCA | 195 |
| Sycp3 | R:TCGCTGTAGACTTCTTGC F:CCGGCTGAGCAAAACTCTAAAAA R:GGAGGCTTTTCATCGCAAC | 161 |

F: forward primer; R: reverse primer
Table S3. Antibodies applied in Western blots

| Antibodies | Source | Dilution |
|------------|--------|----------|
| **Western blots** | | |
| *Dazl* | Abcam cat#ab34139 | 1:1000 |
| *Ddx4* | Abcam cat#ab13840 | 1:3000 |
| *Pllzf* | R&Dsystems cat#MAB2944 | 1:1000 |
| *Sycp3* | R&Dsystems cat#AF3750 | 1:1000 |
| α-Tubulin | Proteintech cat# 66031-1-lg | 1:400 |
| α-Tubulin | Proteintech cat# 11224-1-AP | 1:400 |
| **Secondary Antibodies** | | |
| Donkey anti-Rabbit IgG,Alexa Fluor 488 | Thermo Fisher Scientific cat#A21206 | 1:1000 |
| Donkey anti-Mouse IgG,Alexa Fluor 594 | Thermo Fisher Scientific cat#A21203 | 1:1000 |

5. ELISA Analysis

Blood from the mice in the four groups was collected for ELISA analysis of the levels of T, FSH, and LH via ELISA analysis with ELISA kits (MLBIO, China). Briefly, 10 μL of serum and standard samples was prepared on the test plate and incubated for 30 min at 37 °C; Then, 40 μL of HRP-conjugate reagent was added and incubated with the cells for 60 min at 37 °C. After shaken dry the liquid in the hole, the wells were washed for 30 s five times and incubated for 30 min at 37 °C with 50 μL of a mixture of substrate A and B solutions; finally, 50 μL of stop solution was added to the wells. Finally, the light absorbance was detected by using a spectrophotometer at 450nm. (BioTek, USA).
Figure S1. A~C: Testosterone, LH and FSH expression was tested by ELISA analysis of the four groups 2 weeks after NBMA gavage. The results are presented as the mean ± SD. n = 4 for each group.

6. NMR Spectra
