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Research

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The Effect of Huyang Yangkun Formula on N6-methyladenosine Modification in 4-vinylcyclohexene diepoxide Induced Premature Ovarian Insufficiency Rats

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Abstract

The aging of female reproductive system is mainly manifested by the decline of ovarian function, which is a physiological phenomenon. When it happens prematurely, it is called premature ovarian insufficiency(POI). Huyang Yangkun Formula(HYYKF) was developed according to theory of Chinese medicine and clinical experience and found to obviously relieve symptoms associated with menopause in POI patients. However, little is known about the effect of HYYKF on N6-methyladenosine (m⁶A) modification in ovaries of POI. In this study, VCD induced-POI model was established to investigate the effect of HYYKF on m⁶A modification. We found in the VCD-induced models, protein expressions of the m⁶A enzymes declined except that of FTO, and the expressions of METTL3, METTL14 and YTHDF1 declined very significantly. Interestingly, in naturally aging ovaries, the level of m⁶A declined very significantly at 9-week-old and 24-week-old, and the protein expression of METTL3, METTL14 and FTO in ovaries was down-regulated as mice aged. The data release that the level of m⁶A declines as the ovarian insufficiency occurs, which happens in both naturally aging ovaries and VCD-induced models. We found that HYYKF treatment promoted ovarian follicles development and the level of AMH in VCD induced-POI rats. Most importantly, HYYKF induced expressions of the m⁶A enzymes except that of FTO, and elevated the expressions of METTL3, METTL14, ALKBH5 and YTHDF1 significantly.

Keywords:

premature ovarian insufficiency; Huyang Yangkun Formula; N6-methyladenosine

Introduction

The aging of female reproductive system is mainly manifested by the decline of ovarian function, which is a physiological phenomenon. When it happens prematurely, it is called premature ovarian insufficiency(POI), also called premature ovarian failure, occurs in young women under 40 years old, and has a prevalence rate of 1% (Nelson, 2009; Cooper et al., 2011). The main mechanisms of POI are follicle dysfunction and/or follicle depletion (Huhtaniemi et al., 2018). Due to the loss of ovarian steroid production, it may lead to menopause, impaired fertility, and complications of the skeleton, cardiovascular system, and neuropsychology (Jankowska, 2017). POI is a progressive disease, suggesting that a diagnosis may not mean a termination of ovarian function (De Vos et al., 2010). In the ovaries of POI patients primordial follicles are still present, so conserving these follicles and promoting their development may offer a novel method for preventing POI (Lee and Chang, 2019). Hormone replacement therapy (HRT) is the first-line treatment for POI to relieve symptoms caused by a lack of hormones(Sullivan et al., 2016), however, there is no evidence confirming that HRT promotes follicle development.

Huyang Yangkun Formula (HYYKF) was based on modification of a classic prescription Danggui Buxue Tang, which is widely used for female menopausal diseases (Lin et al., 2017). According to Chinese medicine theory of “Open, Close and Axis of Three Yin and Three Yang”(Yang and Gu, 2016), the core pathogenesis of POI is related to Shaoyin, Jueyin and Taiyin (Yangming). In HYYKF, *Astragalus membranaceus* (Fisch.) Bge. var. *Mongholicus* (Bge.) serves as Jun, promoting the transition of Qi from Shaoyin to Jueyin; *Dioscorea opposita Thunb.* serves as Chen, supplying spleen soil of Taiyin; *Rehmannia glutinosa Libosch.*, *Epimedium brevicornu Maxim.*, and *Cuscuta australis R.Br.* serve as Chen, warming and nourishing Shaoyin kidney essence; *Angelica sinensis* (Oliv.) Diels serves as Zuo, nourishing Jueyin and generating Yang Qi; *Glehnia littoralis Fr.Schmidt ex Miq.* serves as Zuo, promoting the transition of Qi from Taiyin to Shaoyin. Thus HYYKF makes the movement of Qi returns to normal, and makes menstrual regularity, having an ideal effects on POI in clinical applications (Lai and Yang, 2018). In a randomized double-blind controlled

trial, POI patients with liver and kidney deficiency were included(Lai, 2018). It was found that compared with DHEA, HYYKF obviously alleviated menopausal symptoms in patients especially in psychological and urogenital aspects, and significantly improved the level of E₂. In an animal experiment, HYYKF significantly increased the number of antral follicles and mature follicles in 4-vinylcyclohexene diepoxide (VCD) induced POI rats, suggesting the advantages of HYYKF in promoting follicular development and maturity (Xie et al., 2019).

Recently studies have shown that the N⁶-methyladenosine (m⁶A) of messenger RNA is closely related to reproductive function. One study declared that m⁶A was involved in meiosis regulation of the oocyte (Wang et al., 2018; Qi et al., 2016). The m⁶A-methyltransferase methyltransferase-like 3 (Mettl3) was found to increase the proliferation of ovarian granulosa cells(Hua et al., 2018). The m⁶A-reader YT521-B homology (YTH) domain-containing protein 2 (YTHDF2) could regulate oocyte maturation(Ivanova et al., 2017). It seems that m⁶A plays an important role in follicular development. As mentioned previously, HYYKF promotes follicle development, but little is known about whether HYYKF affects the expression of m⁶A modification. So in this study, we focused on the effect of HYYKF on m⁶A modification in 4-vinylcyclohexene diepoxide (VCD) induced POI rats.

Methods

The animals

The research was conducted in accordance with the European Community guidelines (EEC Directive of 1986; 86/609/EEC). Sprague Dawley female rats were supplied by the animal centre of the Southern Medical University (Certification Number: 44007200051804). The rats were housed in a specific-pathogen-free space with 12 hours of alternating light and dark and had free access to food and water. When they were 28 days old, the rats were randomly divided into control (CON), model (MOD), and Huyang Yangkun Formula treatment (HYF) groups. The model was completed by intraperitoneal injection of 160mg/kg VCD (94956, Sigma-Aldrich, Korea) diluted in sesame oil (M07A9E67472, Yuanye Biotech, Shanghai) for 15 days. In the treatment group, the rats were administered with HYYKF 1ml/100g body weight for 75 days

after modelling. The modelling method of VCD was referred to in previous studies (Vabre et al., 2017; Reis et al., 2014). After 90 days, all the rats were euthanised and ovaries and blood samples were obtained. The study was approved by the Guangdong Hospital of Traditional Chinese Medicine Ethics Committees.

Preparation of HYYKF extract

The components of HYYKF were supplied by Kangmei Pharmaceutical Co. Ltd., (Guangdong, China): *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) (171101571), *Angelica sinensis* (Oliv.) Diels (180105951), *Dioscorea opposita* Thunb. (180101909), *Rehmannia glutinosa* Libosch. (180300179), *Epimedium brevicornu* Maxim. (171001721), *Cuscuta australis* R.Br. (180202361), and *Glehnia littoralis* Fr.Schmidt ex Miq. (180205401). The herbs were decocted for one hour with distilled water at ten times volume. The process was repeated twice, and the decoction was mixed together. The solution was rotated to evaporate to the concentration of 1.1 g/ml. The chemical fingerprint HYYKF was analyzed by HPLC and UPLC-MS, and the main active compounds are campanulin, ferulic acid, icariin, formononetin, quercetin, psoralen, and ligustilide (Xie et al., 2019).

Serum analysis

Anti-Müllerian hormone (AMH), follicle stimulating hormone (FSH) levels, estradiol (E2), and luteinizing hormone (LH) levels in serum were determined by ELISA kits (C0100150192, C0100140191, C0100090186, C0100130190, CUSABIO BIOTECH, Wuhan). The hepatic-renal function index [alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (Cr)] was determined in an automatic biochemical analyser (Guangdong Hospital of Traditional Chinese Medicine).

Histology analysis

The ovarian tissue of the rats was immediately fixed with 4% paraformaldehyde. After being dehydrated overnight in an automatic sealed tissue dehydrator, the tissue was paraffin-embedded. Histologic sections were serially sectioned three microns

apart and then stained with hematoxylin and eosin. Follicles were classified as primordial, preantral, antral and mature, and the number of each was determined on every tenth section under a light microscope.

RNA Extraction and measurement of the level of m⁶A in total RNA

To investigate whether m⁶A modification changes with aging, female C57BL/6 mice of 4 weeks, 7 weeks, 9 weeks, and 24 weeks of age were obtained from the animal centre of the Southern Medical University (Certification Number: 11400700277194). The mice were euthanised and the ovaries and hypothalamus were harvested. Total RNA was extracted by TRIzol (182809, Invitrogen) from the ovaries and hypothalamus of all mice. An M⁶A RNA methylation quantification kit (ab185912, Abcam) was used to measure the m⁶A levels in mRNA, following the manufacturer's instructions. The content of m⁶A in each sample was calculated according to the standard curve. The calculation formula is $m^6A\% = [(Sample\ OD - NC\ OD) / S] / [(PC\ OD - NC\ OD) / P] * 100\%$, where S is the ng amount of RNA in samples and P is the ng amount of RNA in positive controls.

Western blot

Protein was extracted from the ovaries and hypothalamus of the mice and the ovaries of the rats by RIPA, the concentration of which was detected by BCA protein assay kit (SE253117A, Thermo Fisher, USA). The protein mixed with 1× loading buffer was denatured at 100°C for ten minutes. SDS-PAGE electrophoresis was performed with 10% separation gel, and then transferred to polyvinylidene fluoride membrane. The membranes were blocked in TBST solution containing 5% BSA for 1.5 hours at room temperature and were incubated overnight at 4°C with the primary antibodies diluted in TBST solution containing 5% BSA (anti-GAPDH Boster BA2913, METTL3 abcam 195352, METTL14 abcam 220030, FTO abcam 92821, ALKBH5 abcam 195377, YTHDF1 abcam 252346, YTHDF2 abcam 220163). The next day the membranes were washed three times, for 10 minutes per wash, and incubated for one hour with the secondary antibody (sheep-anti-rabbit secondary antibody, CST, 7074). The ECL luminescent reagent (Millipore, 1606902) was used to develop the images, and the images were scanned by Western blot automatic imager (ChemiDoc Touch,

Bio-Rad).

Statistical analysis

SPSS 21.0 software was used for data analysis, and the data was expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). One-way analysis of variance (ANOVA) was used for multiple comparisons, while Student's t-test was used for comparison between two groups. $P < 0.05$ was considered to be statistically significant.

Results

Laboratory monitoring of HYYKF

ALT, AST, BUN and Cr in serum were used to monitor the safety of HYYKF in the rats (Table 1). There was no significant difference between the CON and HYF group, indicating that HYYKF has no hepatotoxicity or nephrotoxicity. There was no significant difference between the CON and MOD groups, indicating that VCD has no hepatotoxicity and nephrotoxicity.

Table 1– ALT,AST,ALT/AST,Urea,Cr in rats from CON, MOD and HYF groups.

	CON	MOD	HYF
ALT (U/L)	37 ± 10.2	32.5 ± 2.4	33.75 ± 4.4
AST(U/L)	94 ± 22.6	90.875 ± 16.6	90.375 ± 14.6
ALT/AST	2.5625 ± 0.3	2.7875 ± 0.4	2.7 ± 0.5
Urea(mmol/L)	5.9125 ± 1.1	6.325 ± 0.9	5.3375 ± 0.8
Cr(umol/L)	33.125 ± 4.5	34 ± 4.2	35.375 ± 3.2

Data is shown as mean $\pm$ SD. n=8. There is no significance between CON and MOD group, MOD and HYF group.

Hormone alterations caused by HYYKF

To investigate whether HYYKF alters sex-hormone levels, the levels of AMH, FSH, E2, and LH in serum were assayed (Table 2 and Fig. 1). The level of AMH was

significantly lower in the MOD group than in the CON group ($p < 0.05$). Comparing the HYF group with the MOD group, the level of AMH was increased, but without significance. The difference of FSH, E2, and LH between groups was not significant.

Table 2 –Serum sex-hormone levels in rats from CON, MOD and HYF groups.

	CON	MOD	HYF
AMH (ng/ml)	24.8 ± 7.78	$16.2 \pm 5.69^*$	19.7 ± 5.96
FSH (mIU/ml)	25.0 ± 6.62	28.4 ± 11.00	26.5 ± 8.86
LH (mIU/ml)	5.3 ± 1.17	5.6 ± 1.10	4.4 ± 0.43
E2 (pg/ml)	133.2 ± 34.63	137.1 ± 14.92	120.3 ± 13.30

Data is shown as mean $\pm$ SD. $n=8$, $*P < 0.05$, compared with the CON group.

Fig.1. Levels of AMH, FSH, LH and E2 in serum of rats in the three groups. Data is shown as the mean $\pm$ SD, $*p < 0.05$ compared with the control groups.

Effect of HYYKF treatment on follicular development

Histological analysis of the rat ovaries was performed to assess the effect of HYYKF treatment on ovarian follicle development. Pictures of the ovary sections of the CON, HYF and MOD groups are shown in Fig. 2.A. The follicles were classified according to the following developmental stages: primordial, preantral, antral, and mature; the histological characteristics are shown in Fig. 3. The results are shown as the mean number of follicles at each stage (Table 3). The numbers of follicles at all stages were lower in the MOD group than in the CON group, and the differences in the primordial,

preantral, and antral follicles were especially significant ($P < 0.01$, $P < 0.01$, $P < 0.01$, respectively). In the HYF group, the number of primordial, preantral, antral, and mature follicles was higher than in the MOD group, and the difference in mature follicles was significant ($P < 0.05$)(Fig. 2.B).

Fig.2.A: Ovary sections stained with hematoxylin and eosin of rats in the study: a .CON group; b. MOD group; c. HYF group; B: Number of primordial, preantral, antral, and mature follicles in each group. Data is shown as mean $\pm$ SD. $n=8$, ** $P < 0.01$, compared with the NC group. # $P < 0.05$, compared with the MOD group.

Fig.3.Follicles of different developmental stages: a.primordial follicle; b. preantral follicle; c. antral follicle; d. mature follicle.

Table 3. Number of primordial, preantral, antral, and mature follicles in each group

	CON	MOD	HYF
Primordial	38.6±22.56	12.6±10.06**	20.3±6.89
Preantral	15.1±6.81	5.8±3.63**	7.6±3.00
antral	28.1±5.58	8.1±5.82**	11.4±5.43
mature	3.5±2.60	1.1±1.05	4.3±3.80#

Data is shown as mean ± SD. n=8, **P < 0.01, compared with the NC group. # P < 0.05, compared with the MOD group.

Changes of m⁶A levels and regulatory factors in mice at different ages

To investigate whether m⁶A modification takes place in naturally ageing animals, female C57BL/6 mice of four weeks, seven weeks, nine weeks, and twenty-four weeks of age were studied. The M⁶A levels were determined in the hypothalamus and ovaries of the mice (Fig. 4). The level of m⁶A in the ovaries was highest at seven weeks old, and declined very significantly at nine weeks and twenty-four weeks (P<0.01, P<0.01, respectively). In the hypothalamus the level of m⁶A declined

significantly at nine weeks ($P<0.05$) and, interestingly, increased at twenty-four weeks ($P<0.01$), which was not consistent with the change in ovaries.

Fig.4. The level of m⁶A in ovaries and hypothalamus of mice at different ages. Data is shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, compared with the 7 weeks ovary group or 9 weeks hypothalamus group.

The expression of METTL3, METTL14 and FTO in the ovaries and hypothalamus of mice at different ages are shown in Fig. 5. After a soft increase at nine weeks old, the expression of METTL3 in the ovaries was down-regulated as the mice aged, and the difference between mice at nine weeks old and twenty-four weeks old was especially significant ($P<0.05$). The expression of METTL14 was highest at nine weeks, and there was a very significant difference compared with the four-week old and seven-week old groups ($P<0.01$, $P<0.01$, respectively). The expression of FTO was also highest at nine weeks. The expression of METTL14 and FTO both decreased after nine weeks old. In the hypothalamus, the expression of METTL3, METTL14 and FTO generally tended to be down-regulated as age increased. It was also obvious that at nine weeks old, the expression of METTL14 was most active in both the ovaries and the hypothalamus when the bodies of the mice began to mature.

Fig.5. Images and analysis of protein expression of METTL3, METTL14, FTO in mice. GAPDH expression was used as internal reference. Data is shown as mean $\pm$ SD, *P < 0.05, **P < 0.01.

The effect of HYYKF on protein expressions of m⁶A methyltransferase, demethylase and effector in the rats

To confirm the effect of HYYKF on the expression of m⁶A methyltransferase, demethylase and effector, protein was extracted from the ovaries in each group to perform Western blotting. In the MOD group, the expression of m⁶A methyltransferase METTL3 and METTL14 was significantly decreased compared with those in the CON group (p<0.01, p<0.05, respectively), and the level was significantly increased in the HYF group (P<0.05, p<0.05, respectively) (Fig. 6.A). VCD promoted the expression of RNA demethylase FTO, and the level was decreased in the HYF group. However, another demethylase ALKBH5 went down in the MOD group (Fig. 6.B). Then HYYKF elevated the level of ALKBH5 significantly (P<0.01). As to the m⁶A effectors, VCD intervention decreased the expressions of YTHDF1 and YTHDF2, and HYYKF increased the expressions (Fig. 6.C). The difference of YTHDF1 between the CON and MOD, and MOD and HYF groups, was significant (p<0.01, p<0.05, respectively).

Fig.6. Images and analysis of protein expressions of METTL3, METTL14, FTO, ALKBH5, YTHDF1 and YTHDF2. GAPDH expression was used as internal reference. The protein expression in the CON group was normalized as 1. Relative ratio of MOD group or HYF group to control group was viewed as the index of protein expression quantity. Data is shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, compared with the NC group. # $P < 0.05$, ## $P < 0.01$, compared with the MOD group.

Discussion

There are few studies on the relationship between m⁶A modification and ovarian insufficiency. In this study, we used both naturally aging animals and POI models to explore this subject. Our data suggest that the level of m⁶A declines as ovarian insufficiency occurs, which happens in both naturally aging ovaries and VCD-induced models. Further study indicates that HYYKF affects the expression of m⁶A methyltransferases, demethylases and effectors, and this is probably accounted for HYYKF promotes ovarian follicle development.

To investigate the relationship between m⁶A modification and natural ovarian insufficiency, we chose female mice at four, seven, nine and twenty-four weeks - ranging from adolescence to old age. In the ovaries of the mice, the content of m⁶A in total RNA peaked at seven weeks old, a period of sexual maturity when the ovaries are most active. A study has found that m⁶A methylation was most enriched during the process of follicle selection (Fan et al., 2019), which might indirectly interpret the change of m⁶A modification in our study. It is known that m⁶A is regulated by RNA

methyltransferase, demethylase and effector (Chen et al., 2019). Methyltransferase mainly includes METTL3 and METTL14, and these promote the formation of m⁶A. Meanwhile, m⁶A demethylase α -ketoglutarate-dependent dioxygenase AlkB homolog 5 (ALKBH5) and fat mass and obesity-associated protein (FTO) activate demethylation, which together with methyltransferase make m⁶A dynamic and reversible. In addition, there are some proteins called m⁶A effectors or m⁶A readers, such as YT521-B homology (YTH) domain-containing proteins YTHDF1 and 2, that specifically bind with m⁶A to enable it to function. After nine weeks of age the decreased expressions of METTL3, METTL14 and FTO meant that as ovarian function becomes insufficient, m⁶A modification weakens.

In VCD-induced models, similar results were obtained. VCD is a metabolite of chemical products and is considered to be an appropriate chemical model to simulate the physiological process of ovarian function degeneration in humans (Kappeler and Hoyer, 2012). In the VCD group, the numbers of primordial, preantral, and antral follicles, and the level of AMH in serum, were significantly down-regulated. As is well-known, AMH is a good predictor of ovarian reserve, which reflects the quality and quantity of the remaining follicles in the ovaries (Iwase et al., 2016). Therefore, the falling AMH and follicles number indicated that VCD induced ovarian insufficiency in the rats. The expression of METTL3, METTL14, YTHDF1 and YTHDF2 decreased in the VCD-induced models, which is in line with what we observe in naturally aging ovaries.

Apparently, HYYKF treatment increased the level of AMH and the number of follicles - especially mature follicles - and reversed the situation caused by VCD. As for the regulatory factors of m⁶A, HYYKF induced the expression of m⁶A enzymes (except FTO), and elevated the expression of METTL3, METTL14, ALKBH5, YTHDF1, and YTHDF2. Recent studies have focused on the unique function of the single regulating protein. METTL3 was reported to promote the proliferation of ovarian granulosa cells, and another study also confirmed the promotion role in other cell lines; the mechanism may be that METTL3 helps facilitate DNA damage repair (Xiang et al., 2017). Also, knocking out METTL3 prevented the oocytes from maturing (Xia et al., 2018). METTL14 appears to play a supporting role of stabilizing METTL3 and helping to recognise RNA (Wang et al., 2017). It has been reported that an absence of YTHDF2 results in a failure of oocyte maturation and female fertility (Ivanova et al., 2017). Unlike other regulatory factors, the expression of FTO

increased by VCD and then decreased after HYYKF treatment, which may not be consistent with other studies. One study has reported that after inhibiting the activity of FTO, the proliferation of mouse spermatogonia cells was decreased (Huang et al., 2019). In addition, a case-controlled study showed that the FTO protein expression level was significantly lower in POI patients than in normal people (Ding et al., 2018). However, VCD decreased the expression of ALKBH5, and HYYKF increased the expression. The presence of ALKBH5 ensured correct splicing and the production of longer 3'-UTR mRNAs to ensure spermatogenesis, so the inactivation of ALKBH5 led to male mice sterility (Tang et al., 2018). Moreover, ALKBH5 enhanced the proliferation of ovarian cells, possibly by stabilizing Bcl-2 expression (Zhu et al., 2019). It can be speculated from these findings that the m⁶A regulatory factors play positive roles in the development of ovarian cells and follicles, while in our study HYYKF promotes their expression.

Conclusions

In summary, our study suggests that HYYKF influences the expression of m⁶A ethyltransferases, demethylases, and effectors, and this might account for it promoting ovarian follicle development. The study also indicates that as ovarian insufficiency appears there is a downward trend of m⁶A level and expressions of protein associated with modifications, which happens in both naturally aging ovaries and VCD-induced models. Nevertheless, exactly how m⁶A modification affects follicle development, and the mechanism that underlies the effect of HYYKF on m⁶A in the ovaries, still need to be investigated with further study.

Acknowledgments

Author contributions

H. Y.Y.* conceived and designed the work; M.F.L. wrote the manuscript and performed most part of the experiments; Y.L. performed part of the experiments; J.L. and G.N.N. acquired, analyzed, and interpreted the data; and W.Y.J. participated in data collection and analysis. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Figures

Fig.1.Levels of AMH, FSH, LH and E2 in serum of rats in the three groups. Data is shown as the mean $\pm$ SD , *p < 0.05 compared with the control groups.

Figure 1

Figure legends are viewable on images in this version .

Fig.2.A: Ovary sections stained with hematoxylin and eosin of rats in the study: a .CON group; b. MOD group; c. HYF group; B: Number of primordial, preantral, antral, and mature follicles in each group. Data is shown as mean $\pm$ SD. n=8, **P < 0.01, compared with the NC group. # P < 0.05, compared with the MOD group.

Figure 2

Fig.3.Follicles of different developmental stages: a.primordial follicle; b. preantral follicle; c. antral follicle; d. mature follicle.

Figure 3

Fig.4.The level of m⁶A in ovaries and hypothalamus of mice at different ages. Data is shown as mean $\pm$ SD. *P < 0.05, **P < 0.01, compared with the 7 weeks ovary group or 9 weeks hypothalamus group.

Figure 4

Fig.5.Images and analysis of protein expression of METTL3, METTL14, FTO in mice. GAPDH expression was used as internal reference. Data is shown as mean $\pm$ SD, *P < 0.05, **P < 0.01.

Figure 5

Fig.6.Images and analysis of protein expressions of METTL3, METTL14, FTO, ALKBH5, YTHDF1 and YTHDF2. GAPDH expression was used as internal reference. The protein expression in the CON group was normalized as 1. Relative ratio of MOD group or HYF group to control group was viewed as the index of protein expression quantity. Data is shown as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, compared with the NC group. # $P < 0.05$, ## $P < 0.01$, compared with the MOD group.

Figure 6