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Expression Analysis of *BMPR1B*, *BMP15* and *GDF9* Genes in Polytocous and Monotocous Sheep Breeds During the Luteal Phase

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Authors' Contribution
These studies were designed by KL, JT, XH, YL and MC performed all the experimental analyses and prepared all the figures and tables. KL and JT analyzed the data and drafted the manuscript. YL and MC contributed to revisions of the manuscript. KL, JT, XH and MC assisted in explaining the results and revised the final version of the manuscript.

Key words
***BMPR1B, BMP15, GDF9*, Gene expression analysis, Small tail Han sheep, Sunite sheep, Sheep fertility**

ABSTRACT

To investigate the expression of *BMPR1B*, *BMP15* and *GDF9* genes in sheep at luteal phase, we examined the expression of the three genes in 14 tissues of small tail han sheep (STH) (polytocous breed) and Sunite sheep (SNT) (monotocous breed) by using semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR) and real-time quantitative fluorescence PCR (qPCR) techniques. The results showed that *BMPR1B* was highly expressed in kidney, brain, cerebellum, hypothalamus, pituitary, ovary, uterus and oviduct tissues of STH and SNT sheep, in addition to adrenal gland tissue of STH sheep. *BMP15* was highly expressed in the ovary of STH sheep and in the ovary and oviduct tissues of SNT sheep. *GDF9* was expressed in 14 tissues during the luteal phase in both STH and SNT sheep. These three genes were most highly expressed in ovary tissue from two breeds of sheep. The expression of *BMPR1B* and *GDF9* in ovary tissue of STH sheep was significantly higher than that of SNT sheep ($P<0.01$), while the expression of *BMP15* in ovary tissue of STH sheep was lower than that of SNT sheep, but the difference was not significant. These three genes exhibit varying degrees of expression differences in other non-ovarian tissues along the hypothalamic-pituitary-gonadal (HPG) axis of two sheep breeds. Therefore, the present study suggests that *BMPR1B*, *BMP15* and *GDF9* may play important biological functions in the HPG axis tissues, especially the ovary, during the luteal phase in sheep. These results provide preliminary information on the molecular regulatory mechanisms underlying sheep fertility.

INTRODUCTION

As an important economic animal, the fertility of sheep is important for breed improvement and the development of the farming industry. However, most sheep breeds are monotocous, so effective improvement of sheep reproduction is a pressing problem for the sheep industry. Investigation of the molecular mechanism of fertility is necessary to identify key genes that increase fertility. Among the candidate genes affecting fertility in sheep, bone morphogenetic protein receptor 1B (*BMPR1B*), bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*) have attracted much attention (Akhatayeva *et al*., 2023; Wang *et al*., 2021, 2023; Ji *et al*., 2023). It is known that one or two copies of the *FecB* mutation in *BMPR1B* have an additive effect on ovulation rate and litter size (Akhatayeva *et al*., 2023). The $FecG^{H}$, *FecI* mutants of *GDF9* and the $FecX^{I}$, $FecX^{H}$, $FecX^{G}$, $FecX^{B}$, $FecX^{L}$, and $FecX^{R}$ mutants of *BMP15* are sterile in pure heterozygotes and increase ovulation in heterozygotes (Hanrahan *et al*., 2004; Nicol *et al*., 2009; Roy *et al*., 2011; Estienne *et al*., 2017), whereas the $FecG^{E}$ mutant of *GDF9* increases ovulation rate only in the homozygote (Silva *et al*., 2011). In addition, *BMPR1B*, *GDF9* and *BMP15* are all family members of the TGF-β pathway and studies have shown that they are closely related and work together to regulate and influence ovulation and lambing in sheep (Crawford *et al*., 2011; Song *et al*., 2023; Çelikeloğlu *et al*., 2021).

The luteal phase is the period between the

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transformation of the dominant follicle into the corpus luteum shortly after ovulation and the establishment of pregnancy or the onset of menstrual bleeding (Stocco *et al*., 2007). During the luteal phase, the corpus luteum secretes progesterone and a number of other hormones that are essential for maintaining the endometrium in a condition conducive to embryo attachment and development (Devoto *et al*., 2009). To date, most studies of polytocous candidate genes such as *BMPR1B*, *BMP15* and *GDF9* have focused on correlation analysis between mutations at the locus and litter size, while fewer comparative analyses of tissue-specific expression of major organs, glands, and reproductive axes have been reported in polytocous and monotocous sheep breeds (Song *et al*., 2023; Ji *et al*., 2023), especially during the luteal phase. STH sheep and SNT sheep are two indigenous Chinese sheep breeds. STH sheep is a remarkable polytocous sheep breed in northern China known for its early maturity, year-round estrus and prolific production (Li *et al*., 2023). SNT sheep is a representative local fine breed in Inner Mongolia, China, which is characterized by cold resistance, drought resistance, rapid growth and development, high vigor, delicate meat and good flavor. However, it is a monotocous sheep breed (He *et al*., 2022). Therefore, in this study, we investigated the expression of *BMPR1B*, *BMP15* and *GDF9* in major organs and gonadal axis tissues of STH sheep and SNT sheep during the luteal phase. The results of this study may provide a reference for improving the reproductive performance of sheep.

MATERIALS AND METHODS

Animal and sample collection

In this study, six healthy ewes (three each of STH and SNT ewes) aged 2-3 years were randomly selected from sheep farms of Tianjin Animal Science Research Institute as test material. The oestrous cycles of all ewes were synchronized with the progesterone vaginal embolism (CIDR, New Zealand, InterAg Co., Ltd.) inserted into the vagina for 12 days. Heart, liver, spleen, lung, kidney, brain, cerebellum, hypothalamus, pituitary, ovary, oviduct, uterus, adrenal, duodenum tissues were collected from selected STH and SNT ewes 10 days after CIDR removal. Tissue samples are collected within 30 minutes of euthanasia, rapidly frozen in liquid nitrogen and stored in liquid nitrogen for further use.

Primer design

Based on the sequences of the sheep reference genes *BMPR1B* (NM_001009431.1), *BMP15* (NM_001114767.1), *GDF9* (NM_001142888.2) and *β-actin* (NM_001009784.1) from GenBank (http://www.ncbi.nlm.nih.gov/). The primers were designed using the Premier 5.0 software for sqRT-PCR and qPCR specific primers (Table I). Primer design was completed and sent to Tianyi Biotechnology Co Ltd (Beijing, China) for synthesis.

Table I. Primers used in the present study.

Methods	Gene	Primer sequence	Length (bp)	Tm (°C)
sqRT-PCR	*BMPR1B*	F: 5'-GGGTTCTACGACTCCGCTTC-3' R: 5'-GGTTACTTTCAGGCCCATCAT-3'	237	60
	BMP15	F: 5'-GGGTTCTACGACTCCGCTTC-3' R: 5'-GGTTACTTTCAGGCCCATCAT-3'	273	62
	GDF9	F: 5'-TAGTCAGCTGAAGTGGGACA-3' R: 5'-AGCCATCAGGCTCGATGGCC-3'	224	61
	β-actin	F: 5'-ACCCAGCACGATGAAGATCA-3' R: 5'-GTAACGCAGCTAACAGTCCG-3'	187	61
qPCR	*BMPR1B*	F: 5'-TGACGGACCTATACACCACA-3' R: 5'-GTACCGAGGTCTGGCTTCTT-3'	121	60
	BMP15	F: 5'-TGTTGGGCAAAAGCTCTGGA-3' R: 5'-GCCATGCCACCAGAACTCAA-3'	106	60
	GDF9	F: 5'-AACAGACGCCACCTCTACAA-3' R: 5'-CACGATCCAGGTTAAACAGCA-3'	124	60
	β-actin	F: 5'-ACCCAGCACGATGAAGATCA-3' R: 5'-GTAACGCAGCTAACAGTCCG-3'	97	60

Total RNA extraction and cDNA synthesis

Tissue RNA was extracted using an animal tissue total RNA extraction kit (Tiangen Biotechnology Co., Ltd., Beijing, China). RNA integrity was detected by agarose gel electrophoresis and concentration was determined using a NanoDrop 2000 spectrophotometer, and cDNA was obtained by reverse transcription according to the instructions of the reverse transcription kit and then stored in a refrigerator at -20 °C for subsequent experiments.

sqRT-PCR program and amplification system

The reverse transcribed cDNA was used for sqRT-PCR analysis. The volume of the amplification system of RT-PCR was 20 μL, containing 10 μL of 2×PCR Master Mix (Biomed, Beijing, China), 0.5 μL of 10 μM/L primer (forward and reverse), 1.0 μL of cDNA and 8 μL of ddH_2O. The amplification program was: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing temperature (Table I) for 30 s, and extension at 72 °C for 60 s. The cycles for *BMPR1B*, *BMP15*, and *GDF9* are 32, 36, and 34, respectively, with a final extension at 72 °C for 5 min.

System and program of Real-time qPCR

The real-time PCR amplification reaction mixture (20 μL) contained 10 μL SYBR® Premix ExTaq II, 0.8 μL forward and reverse primer (10 μM), 2 μL cDNA, and 6.4 μL ddH_2O. Reactions without template were used as blanks. The program was carried out under these conditions, initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 5 s and 60 °C for 30 s. After amplification, the melting curve was analyzed. Real-time qPCR was performed in triplicate with negative controls (H_2O as template) on the Roche Light Cycler®480°C. Gene expression data were normalized to housekeeping gene (*β-actin*) data.

Statistical analysis

The relative gene expression level was calculated by $2^{-\Delta\Delta Ct}$ method. Gene expression differences between different tissues or breeds were analyzed using SPSS software with one-way analysis of variance method. Multiple comparisons were performed using the least significant difference (LSD) method.

RESULTS

Tissue expression profiling of BMPR1B, BMP15 *and* GDF9 *genes*

After sqRT-PCR amplification, 1.5% agarose gel electrophoresis showed that the products of *BMPR1B*, *BMP15*, *GDF9* and *β-actin* were of the expected fragment length. Their expression in fourteen tissues of STH and SNT sheep is shown in Figure 1. *BMPR1B* was highly expressed in kidney, brain, cerebellum, hypothalamus, pituitary, ovary, uterus and oviduct tissues of STH and SNT sheep. In addition, *BMPR1B* was highly expressed in adrenal gland tissue of STH sheep. *BMP15* was highly expressed in the ovary tissue of STH sheep. However, it was highly expressed in the ovary and oviducts of SNT sheep. In addition, *GDF9* was expressed in fourteen tissues from two breeds of sheep.

Fig. 1. Tissue expression profile of *BMPR1B*, *BMP15*, *GDF9* and *β-actin* genes in (A) STH sheep and (B) SNT sheep breeds.
Lanes: 1-14: heart, liver, spleen, lung, kidney, brain, cerebellum, hypothalamus, pituitary, ovary, uterus, oviduct, adrenal gland and duodenum, respectively. M: DL2000 DNA Marker.

Expression levels of BMPR1B *gene in the HPG axis*

As shown in Figure 2A, the expression of *BMPR1B* was highly significantly higher in the ovary tissues of STH sheep than in the other four tissues ($P<0.01$), and it was highly significantly higher in the hypothalamus and oviduct tissues than in the pituitary and uterus tissues ($P<0.01$), and it was significantly higher in the oviduct tissues than in the hypothalamus ($P<0.05$). The expression of *BMPR1B* was highly significantly higher in the ovary, hypothalamus and oviduct tissues than in the pituitary and uterus of SNT sheep ($P<0.01$). In addition, as shown in Figure 2B, the expression of *BMPR1B* was highly significantly higher in ovary and uterus tissues of STH sheep than that of SNT sheep, but was highly significantly lower in pituitary tissues than that of SNT sheep ($P<0.01$), and there was no significant difference in hypothalamus and oviduct tissues ($P>0.05$).

Expression levels of BMP15 *gene in the HPG axis*

As shown in Figure 2C, the expression of *BMP15*

was highly significantly higher in ovary tissue of STH sheep than in the other four tissues (P<0.01), but the difference was not significant between hypothalamus, pituitary, oviduct and uterus tissues (P>0.05). It was highly significantly higher in ovary and oviduct tissues of SNT sheep than in hypothalamus, pituitary and uterus tissues (P<0.01), but the differences were not significant between ovary and oviduct tissues and between hypothalamus, pituitary and uterus tissues (P>0.05). In addition, as shown in Figure 2D, the expression of *BMP15* was significantly higher in the hypothalamus and pituitary tissues of STH sheep than that of SNT sheep (P<0.05), and was highly significantly lower in the oviduct tissues than that of SNT sheep (P<0.01), but the difference was not significant in the ovary and uterus tissues (P>0.05).

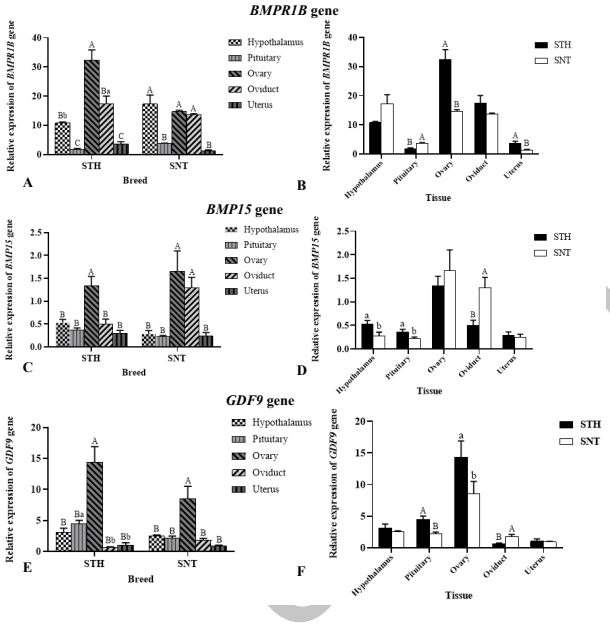

Fig. 2. The relative expression of *BMPR1B*, *BMP15* and *GDF9* genes. (A, C, E) gene expression in two sheep breeds; (B, D, F) gene expression between tissues.
Note: Comparison within breeds and tissues. The expression levels with the same or no letters have no significant difference (P>0.05). The expression levels with the different small letters have significant difference (P<0.05), the expression levels with the different capital letters have highly significant difference (P<0.01).

Expression levels of GDF9 *gene in the HPG axis*

As shown in Figure 2E, the expression of *GDF9* was highly significantly higher in ovary tissues of STH and SNT sheep than in the other four tissues (P<0.01), and also significantly higher in pituitary tissues than in oviduct and uterus of STH sheep (P<0.05), but there was no significant difference between hypothalamus, pituitary, oviduct and uterus tissues of SNT sheep (P>0.05). In addition, as shown in Figure 2F, the expression of *GDF*9 was highly significant in pituitary tissue of STH sheep (P<0.01) and significantly higher in ovary tissue (P<0.05) than SNT sheep, but significantly lower in oviduct tissue (P<0.05) than SNT sheep, whereas there was no significant difference in hypothalamus and uterus tissues (P>0.05).

DISCUSSION

Function and expression of BMPR1B *gene*

Since the discovery of the *FecB* mutation in Booroola sheep, *BMPR1B* has been identified as an important gene influencing litter size in sheep (Gao *et al*., 2021). *BMPR1B* is a member of the TGF-β receptor family and is located on sheep chromosome 6 (Montgomery *et al*., 1994). Studies have shown that *BMPR1B* may influence the differentiation of granulosa cells and the maturation of ovulatory follicles (Mulsant *et al*., 2001). It may therefore be related to the rate of ovulation and multiple births in ewes. *BMPR1B* is a membrane receptor for bone morphogenetic proteins, which are widely expressed in various tissues of the body (Song *et al*., 2023). Studies have shown that *BMPR1B* is highly expressed in the brain and reproductive tissues of sheep (Wen *et al*., 2021; Tang *et al*., 2018). And *BMPR1B* was found to be expressed and play an important role in goat luteal tissue (Silva *et al*., 2005). In this study, the *BMPR1B* was expressed in most of the luteal phase tissues of both breeds of sheep and was most highly expressed in ovary tissues. This is similar to the findings of Wen *et al*. (2021) in STH sheep. This finding suggests that it may play an important role in the normal physiological function of ovary tissue, as well as other tissues and organs.

The expression of *BMPR1B* in this study was significantly higher in STH ovary and uterus tissues and significantly lower in pituitary tissue than in SNT sheep. It is well known that the BMP/Smad signaling pathway is involved in follicular development, ovulation, and female reproduction, especially fertility, in domestic animals (Abdurahman *et al*., 2019). And the high expression of *BMPR1B* in the tissues of polytocous sheep breeds may better regulate the relevant cytokines in the BMP/Smad signaling pathway and thus improve the ovulation rate (Xu *et al*., 2010). Our results support this view. Therefore, the present study suggests that the high expression of *BMPR1B* in ovary tissue may have an important biological function for follicular development in sheep and is a master gene affecting lambing performance.

Function and expression of BMP15 *gene*

BMP15 is essential for normal follicular development and is a member of the TGF-β superfamily associated with

fertility in sheep (Juengel *et al*., 2002). *BMP15* is located on the sheep X chromosome and consists of two exons and one intron. *BMP15* is primarily expressed in oocytes and promotes granulosa cell proliferation and ovarian mound expansion (Christoforou and Pitman, 2019; Taheri *et al*., 2021). *BMP15* was found to be expressed in mouse pituitary tissue (Otsuka and Shimasaki, 2002) and was highly expressed in sheep ovary and trace expressed in sheep spleen, lung, kidney, hypothalamus and oviduct (Tang *et al*., 2018). In this study, we found that *BMP15* was highly expressed not only in the ovary of two breeds of sheep, but also in other tissues such as hypothalamus and pituitary to varying degrees. Previous studies have shown that *BMP15* maintains the low incidence of cumulus cell apoptosis by establishing a local gradient of bone morphogenetic proteins (Hussein *et al*., 2005). Thus, based on the high expression of *BMP15* in ovary tissue during the luteal phase in sheep, it may be an important factor in maintaining numerous follicular developments and may play an important role in ovarian function.

Previous studies have shown that *BMP15* is expressed at significantly lower levels in oocytes from mutant (high fertility) sheep than in wild-type (low fertility) sheep (Crawford *et al*., 2011). In addition, the expression of *BMP15* in the ovary of goats from high fertility breeds was significantly higher than that in the ovary of goats from low fertility breeds (Cui *et al*., 2009). The expression of *BMP15* in luteal phase tissues of sheep has not been reported. In the present study, there was no significant difference in the expression of *BMP15* in luteal phase ovary tissues of sheep with different lambing performance, but the expression of *BMP15* in hypothalamus and pituitary tissues of STH sheep was significantly higher than that of SNT sheep. The results of the present study are inconsistent with the conclusions of previous studies, and the analysis of the reasons may be mainly related to the different periods of tissue sampling as well as the differences in species, whereas the differences in expression in hypothalamic and pituitary tissues may be related to the effects of hormones or regulatory factors.

Function and expression of GDF9 *gene*

GDF9 has been identified as a member of the TGF-β superfamily. The gene is located on sheep chromosome 5, spans approximately 2.5 kb, and has two exons and one intron (Sadighi *et al*., 2002; Aboelhassan *et al*., 2021). This gene plays an important role in early folliculogenesis in female reproduction as a growth and differentiation factor secreted by mammalian oocytes (Elvin *et al*., 1999). *GDF9* has been found to be expressed in oocytes and persists during follicular maturation and ovulation (Dube *et al*., 1998), indicating that it may play an important role in oocyte maturation. It has been shown that *GDF9* expression in rodents is not restricted to the ovary, but is also present in the testis and hypothalamus. As with mRNA expression in the ovary, mRNA expression in the testis is largely restricted to germ cells. In addition, *GDF9* has been shown to be expressed in the human ovary and testis, as well as in a variety of non-reproductive tissues (Fitzpatrick *et al*., 1998). And *GDF9* is also expressed in the luteal tissue of goats (Silva *et al*., 2005). *GDF9* is expressed in multiple tissues of sheep, and its expression in the ovary is significantly higher than that in other tissues (Pan *et al*., 2018). The present study also found that the *GDF9* was expressed in tissues sampled during the luteal phase in both species of sheep, with the highest expression in ovary tissues. This suggests that it plays an important role not only in the reproductive organs, but also in other organs and tissues.

It has been reported that differential expression of *GDF9* may be an important basis for high productivity in goat breeds. Because the expression of *GDF9* was significantly higher in the small antral follicle of polytocous Black Bengal goats than in monotocous Sirohi goats (Pramod *et al*., 2013). In the present study, *GDF9* gene expression was higher in ovarian and pituitary tissues during the luteal phase in STH sheep than in SNT sheep, whereas it was lower in oviduct tissues than in SNT sheep. *GDF9* acts synergistically with hormones or growth factors such as FSH and *BMP15* during follicular development in animals (Vitt *et al*., 2000). Therefore, it is speculated that differential expression of *GDF9* in the non-ovarian tissues of the HPG axis may be associated with certain hormones and growth factors and thus affect follicular development and ovulation in sheep. The high expression of *GDF9* in ovary tissue of polytocous sheep suggests that it may have a significant effect on follicular development and is the major gene affecting lambing performance.

The interaction of BMPR1B, BMP15 *and* GDF9

BMPR1B, *BMP15* and *GDF9* are all expressed in a variety of tissues and are strongly associated with reproductive performance in female animals. Important interactions between *BMPR1B*, *BMP15* and *GDF9* during follicular development influence lamb performance in sheep. Studies have already shown that combinations of these mutations act synergistically and that fertility may be affected in some animals carrying multiple mutations (Hanrahan *et al*., 2004; Su *et al*., 2004). In addition, *GDF9* and *BMP15* synergistically regulate mammalian granulosa cell proliferation and gonadotropin-induced differentiation (Mcnatty *et al*., 2005a, b), and oocyte maturation (Kathirvel *et al*., 2013). *GDF9* and *BMP15* are growth factors secreted by oocytes (Mottershead *et al*., 2012; Heath *et*

al., 2017) and both utilize receptor complexes composed of two types of serine-threonine kinases. *BMPR2* is a type 2 receptor used by *GDF9* and *BMP15*, with *GDF9* using *TGFBR1* (*ALK5*) and *BMP15* using *BMPR1B* (*ALK6*) as a type 1 receptor (Juengel *et al*., 2013). The *FecB* mutation is located in *BMPR1B*, which may explain the synergistic effect between *BMPR1B* mutation and *BMP15* mutation. In addition, *ACVR1B* may be involved in signaling when *BMP15* and *GDF9* are present at the same time (Peng *et al*., 2013). The classical signaling pathway used by these receptors is the SMAD pathway with two receptor-related subfamilies (Miyazawa *et al*., 2002; Ten-Dijke and Hill, 2004). In summary, the three genes, *BMPR1B*, *BMP15* and *GDF9*, have an interactive and synergistic relationship and jointly regulate important reproductive physiological processes such as ovarian development and follicular maturation. In sheep luteal ovary tissue, *BMPR1B*, *BMP15* and *GDF9* were all highly expressed in this study. Their synergistic effects during the luteal phase in sheep require further study.

CONCLUSION

This study found that *BMPR1B*, *BMP15* and *GDF9* are all highly expressed in the ovary tissue during the luteal phase in sheep, and have varying degrees of expression in other tissues, indicating that these three genes have extensive biological functions and play important roles in the ovary. *BMPR1B* and *GDF9* are significantly highly expressed in the ovary of polytocous sheep breeds, which are the main genes affecting lambing performance, while *BMP15* is not significantly expressed, and may affect sheep reproductive performance by regulating other traits.

DECLARATIONS

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Ethical statement and IRB approval

The Science Research Department (in charge of animal welfare issues) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS; Beijing, P. R. China) has approved all procedures involving laboratory animals. Ethics approval was also granted by the animal ethics committee of IAS-CAAS (No. IASCAAS-AE-03; December 12th, 2016).

Generative AI and AI-assisted technology statement

The authors have declared that no generative AI or AI-assisted technologies were used to create this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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