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BMP4 gene expression in native goat breeds in Kerala

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Abstract
Bone morphogenetic proteins (BMPs) are multifunctional cytokines belonging to the transforming growth factor family and play a vital role in early embryonic development, tissue homeostasis and inhibition of progesterone synthesis. It was also reported to have an effect on skeletal development and adipogenesis regulation. The present study was undertaken to compare the expression of the BMP4 gene in the ovary and the uterus of Malabari and Attappady Black goats. Samples were collected from the uterus and the ovary from the abattoir and the RNA was isolated. The isolated RNA was converted to cDNA and was used for real-time PCR analysis with GAPDH as reference gene. The ovary of Attapady Black goat breed had a 0.26-fold lower expression of BMP4 than that in the ovary of the Malabari goat breed (p<0.05). Statistical analysis revealed a 0.13-fold decrease of BMP4 expression in the ovary compared to the uterus (p<0.05). A significant difference was observed in the expression of BMP4 gene between tissue samples (uterus and ovary) as well as between the two breeds (p<0.05).

Keywords: BMP4, Malabari, Attappady Black, PCR, mRNA, qRT-PCR

1. Introduction
Goat occupies a special niche in the Indian agricultural production system, as it is a multi-purpose animal. It produces meat, milk, skin and hair and can utilize poor-quality grass and crop residues. Due to their small size they could be reared by women and children and can sustain in areas with limited natural resources (Formiga de Sousa *et al*. 2015; Gobba *et al*. 2014) [6, 7]. The goat population of India in 2019 was 148.88 million with a growth of 10.14 per cent over the previous census (Livestock census, 2019) [12]. As per the National Bureau of Animal Genetic Resources, there are 37 recognized goat breeds in India.
Kerala has two native breeds of goat, *viz*. Malabari and Attappady Black goats. Malabari breed is dual-purpose breed and highly prolific in nature. Adult females weigh 34.25±0.85kg while the males weigh 43.63±2.51kg (Verma *et al*. 2009) [18]. The Attappady Black goats originated from the hilly regions of Attappady in Palakkad. They are extremely hardy and more disease resistant than other breeds in Kerala. It is a meat breed and has an adult body weight of 34.47kg for males and 31.31kg for females (Stephen *et al*. 2005) [14].
Bone morphogenetic proteins (BMPs) are multifunctional cytokines that belong to the transforming growth factor ß superfamily. They have been reported to play diverse roles in embryogenesis, regulation of adipogenesis (Elsen *et al*. 2014) [4] and energy homeostasis (Katagirir and Watabe, 2010) [10]**.** At present more than 30 members of the BMPs have been identified out of which BMP4 is one of the most important proteins. It has also been reported to play a role in skeletal development and bone formation by regulating vascular and valvular calcification (Hogan 1996; Bellusci 1996) [8,1]. Timothy *et al*., in 2004 [17] determined its role in bone mass, structure, and possibly bone strength resulting in the differentiation of human embryonic stem cells to trophoblast.
Expression of BMP4 was observed to be higher in healthy follicles compared to those undergoing atresia (Shimasaki *et al*. 1999) [15]. Previous studies in sheep (Juengel *et al*. 2006) [9] and cattle (Fatehi *et al*. 2005) [5] have indicated the role of BMP4 in the inhibition of progesterone production and decrease in basal granulosa cells progesterone secretion thereby preventing FSH stimulating action.
In view of the above observations, the present study was undertaken to analyse the expression profile of the BMP4 gene in ovarian and uterine tissue samples of Malabari and Attappady Black goats.

2. Materials and Methods

2.1 Isolation of RNA: Tissue samples from the uterus and the ovary were collected following standard protocol and frozen in RNA later (Sigma-Aldrich) at -40ºC from six goats each of Malabari and Attappady Black goats slaughtered at the University Meat Plant, Thrissur. Gen Elute mammalian total RNA miniprep kit (RTN70, Sigma Aldrich), was used to isolate RNA from about 100mg of tissue samples following necessary precautions. The quality and concentration of the extracted RNA was checked using Nanodrop TM 1000 spectrophotometer (Thermo Scientific, USA).

2.2 cDNA Synthesis

Samples having good quality and concentration were selected and converted to cDNA using Takara Prime Script 1st strand cDNA Synthesis Kit. The reaction mixture comprised of 5µL of RNA, 1µL of random primer, 1µL of dNTP and RNAse free water to make the total volume to 12.5 µL. The mixture was incubated at 65ºC for 5 min followed by snap chilling on ice. From this reaction 10µL was taken and added to a PCR tube containing 4 µL of 5X primer buffer, 0.5 µL of RNAse inhibitor, 1.0 µL of Primescript RTase and 4.5 µL of RNAse free water. Incubation conditions comprised of 30ºC for 10 min, 42 ºC for 45 min and 95ºC for 5 minutes followed by cooling on ice.

2.3 qRT-PCR

Primers for BMP4 and GAPDH were designed using sequences from NCBI using Primer3 software and Primer stat. The sequence and properties of the designed primers for BMP4 and GAPDH gene are presented in Table 1. Gradient PCR was performed to identify optimal annealing temperature and the reaction mixture was prepared as per Table 2. The composition of reaction mixture used for PCR is presented in Table 3 and the PCR programmes used are presented in Table 4.

Table 1: Sequences and properties of primers designed for qRT-PCR

Primer	Primer sequence (5'-3')	Accession No.	Length	GC%	Product size
BMP4QF	AGCTTCCACCACGAAGAACA	XM_018053542.1	20	50	95bp
BMP4QR	TTCTCTGGGATGCTGCTGAG		20	55	
GAPDHF	TGGAGAAACCTGCCAAGTATG	XM_005680968.3	21	47	127bp
GAPDHR	TGAGTGTCGCTGTTGAAGTC		20	50	

Table 2: PCR reaction mixture used to amplify BMP4 and GAPDH *gene* fragments for standardization

Sl. No.	Item	Quantity (µL)
1.	Nuclease free water	3.9
2.	2 X PCR master mix (Emerald Amp® GT PCR Master Mix)	5.0
3.	Forward Primer (10 µM/ µL)	0.3
4.	Reverse Primer (10 µM/ µL)	0.3
5.	Template	0.5
6.	Total	10.0

PCR products (4µL) were loaded in two percent agarose gel, alongside Gene Ruler (Thermo Scientific) 100 bp DNA Ladder. Electrophoresis was carried out at 80V for 30 minutes. Using a gel documentation system (Bio-Rad), the gels were visualized. under UV light and photographed.

Using the GAPDH gene as the reference gene, real-time PCR was performed using Illumina Eco® qRT-PCR system. The reaction mixture for qRT-PCR and the standardized qRT-PCR are provided in Table 3 and 4 respectively. The expression of BMP4 gene was calculated using $2^{-\Delta\Delta CT}$ procedure [11] (Livak and Schmittgen, 2001) [11].

Table 3: Reaction mixture for qRT-PCR

Sl. No	Component	Volume (µl)
1.	Maxima SYBR Green qPCR Master Mix	6.25
2.	Forward primer (10 pM/µl)	0.8
3.	Reverse primer (10 pM/µl)	0.8
4.	Template (cDNA)	2.0
5.	Nuclease free water	2.65
	Total	12.5

Table 4: qRT-PCR conditions to amplify BMP4, and GAPDH genes

Sl. No.	Step	Genes	
		BMP4	GAPDH
		95bp	127bp
1.	Initial denaturation	95 ºC (5 min)	95 ºC (5 min)
2.	Denaturation	95 ºC (30s)	95 ºC (30s)
3.	Annealing	60.7 ºC (30s)	62.2 ºC (30s)
4.	Extension	72 ºC (30s)	72 ºC (30s)
5.	Final extension	72 ºC (5 min)	72 ºC (5 min)
Steps 2-4 were repeated for 40 cycles			

3. Results and Discussion

The mean ratios obtained at 260/280 nm and 260/230 nm were 2.02±0.17 and 2.13±0.15, respectively which was in accordance with previous literature published (Desjardins and

Conklin 2010) [3]. A single peak indicated no contamination with DNA or proteins (Fig 1.) As the OD values are not always an accurate indicator of RNA quality, agarose gel electrophoresis was used to check the integrity of the extracted RNA (Bustin 2000). [2] Distinct bands of 28 S and 18 S rRNA indicated the high quality of the isolated RNA. Light smearing observed between these two bands depicted the presence of mRNA (Fig 2).

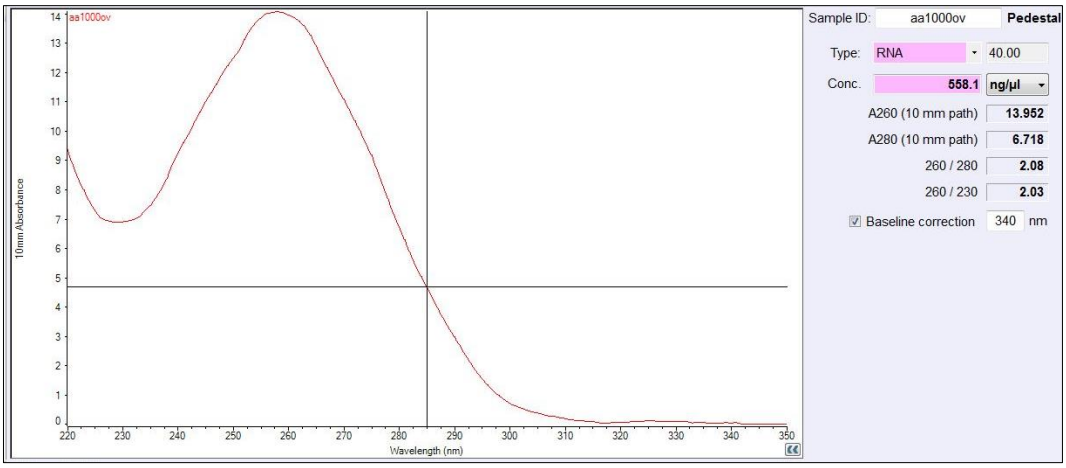

Fig 1: Concentration and optical density of RNA measured in NanoDrop spectrophotometer

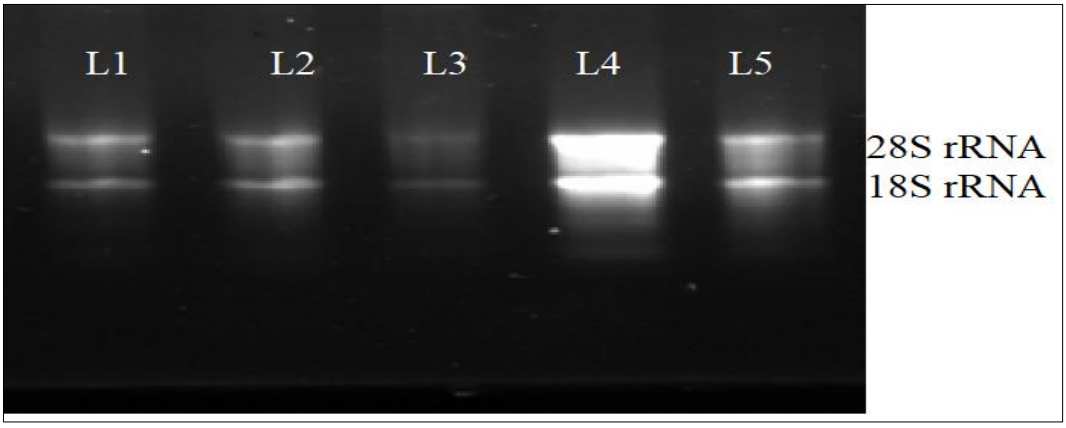

Fig 2: RNA from the tissue samples of ovary and uterus

The PCR conditions were standardized during a gradient PCR for the target and reference genes. The reference gene should show stable C_t values and constant expression in the tissues being used for the experiment and based on previous literature Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* gene was selected as the endogenous control (Naicy,2015; Winer *et al*., 1999) [13,19]. Final products were visualized on a two percent agarose gel stained with ethidium bromide.

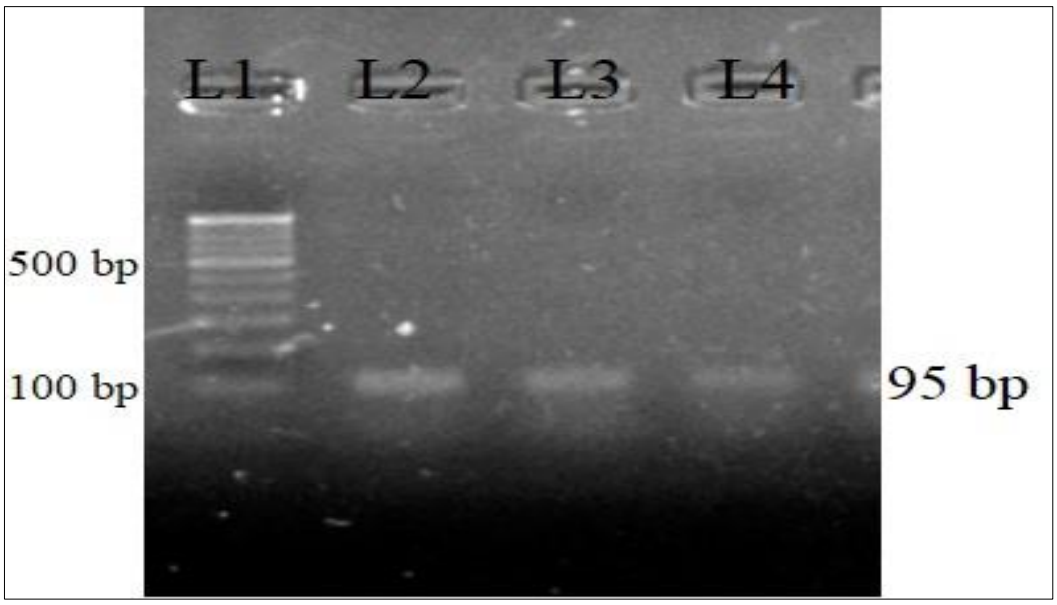

Fig 3: Lane 1: 100bp ladder, Lanes 2-4: 95bp product of BMP4 gene

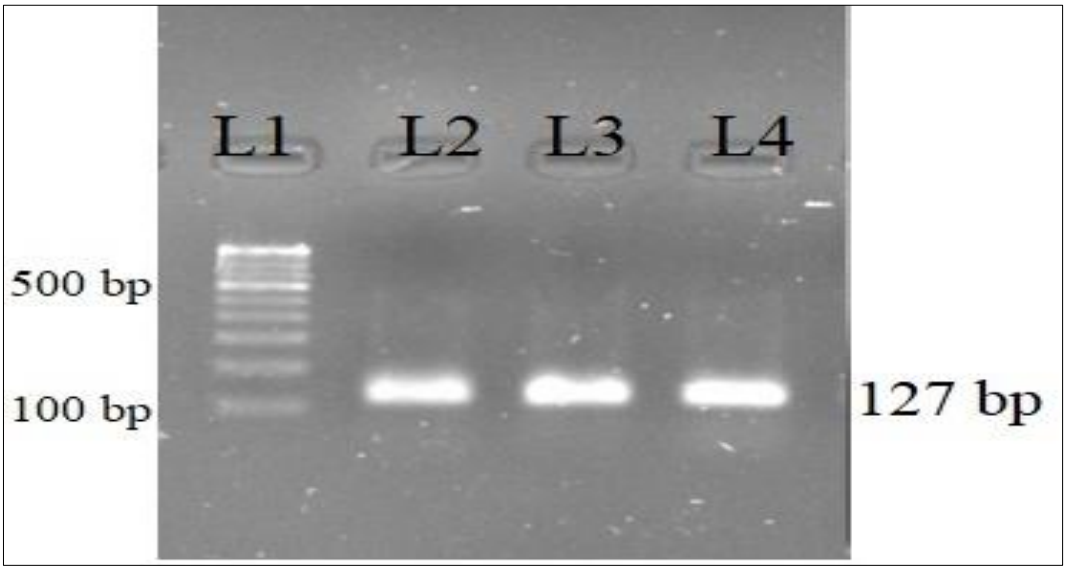

Fig 4: Lane 1: 100bp ladder, Lanes 2-4: 127bp product of *GAPDH* gene

The presence of a single peak during real-time PCR indicated the absence of primer dimers and non-specific products (Fig 5). The amplification plots are presented in Fig 6. The C_t mean values were calculated from the data obtained from the Illumina Eco® qRT PCR for Malabari and Attappady Black goat breeds. The ovary of the Attapady Black goats had a 0.26-fold decrease in the expression of BMP4 compared to the ovary of the Malabari goat breed. Lower expression of BMP4 of 0.36-fold was observed in the uterus of the Attappady Black goat breed compared to Malabari. A significant difference in the expression of BMP4 was observed between the ovaries of the Malabari and Attappady Black goats.

A significant difference was also observed in the expression of BMP4, between the ovary and the uterus of the two breeds (p<0.05). Tissue wise comparison was also performed. The tissue wise comparison revealed that mRNA levels of BMP4 gene in the uterus and ovary differed significantly (p<0.05). A 0.13-fold decrease was observed in the expression of BMP4 in the ovary compared to the uterus. The graphs for the breed wise and tissue wise comparison are depicted in Fig 7 and Fig 8.

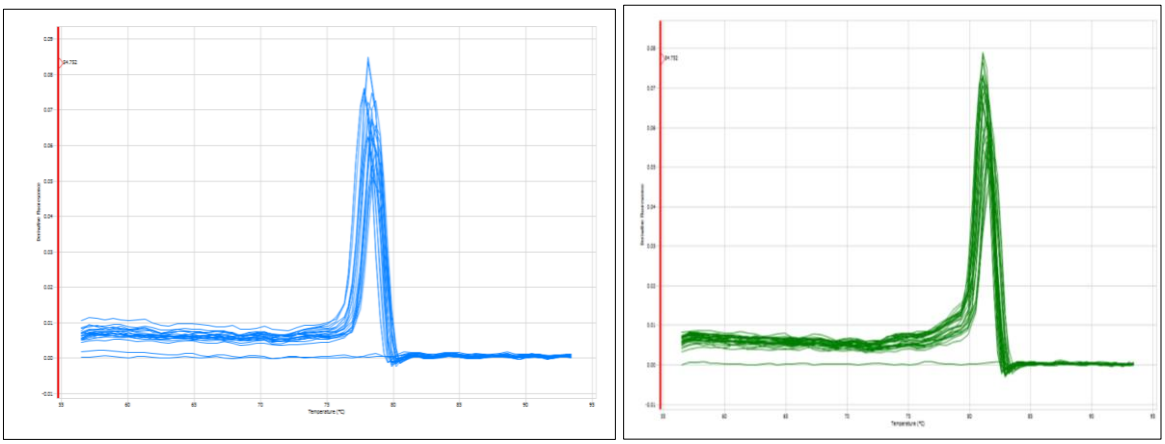

Fig 5: Melt curve for BMP4 and *GAPDH* genes

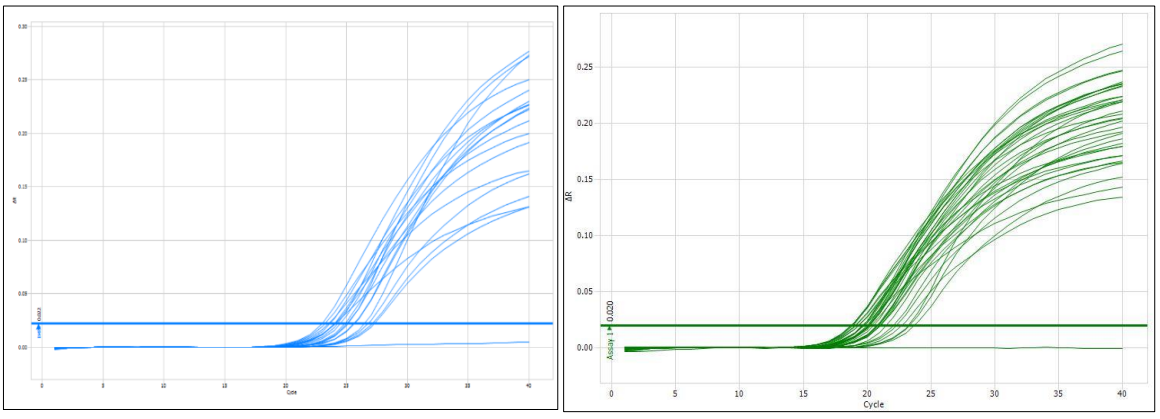

Fig 6: Amplification graph for BMP4 and *GAPDH* genes

Table 5: Relative expression profile of BMP4 gene in the ovary and the uterus tissues of Attappady Black (AB) and Malabari (M) goats

Tissue	Breed	Mean C_T±SE		Δ C_T±SE	ΔΔ C_T±SE	Fold change from control ($2^{-\Delta\Delta C_T}$)	P value (≤ 0.05)
		BMP4	*GAPDH*				
Ovary (Control)	M	26.12±0.70	20.01±0.83	6.1 ±0.78	0±0.78	1	0.03
	AB	28.55±1.41	20.53±0.73	8.02 ±1.01	1.92 ±1.01	0.27	
Uterus (Control)	M	23.02±0.83	19.62±0.60	3.40± 0.27	0 ±0.27	1	0.03
	AB	26.16±0.98	21.27±0.81	4.89±0.48	1.49 ±0.48	0.36	

Table 6: Relative expression profile of BMP4 gene in the tissues of ovary and uterus

Tissue	Mean C_T± SE		ΔC$_T$± SE	ΔΔC$_T$± SE	Fold change from control ($2^{-\Delta\Delta C_T}$)	P value (≤ 0.05)
	BMP4	*GAPDH*				
Ovary (Control)	24.59± 0.86	20.45± 0.61	4.15±0.72	0±0.72	1	0.02
Uterus	27.33± 0.72	20.27± 0.52	7.06±0.35	2.91±0.35	0.13	0.04

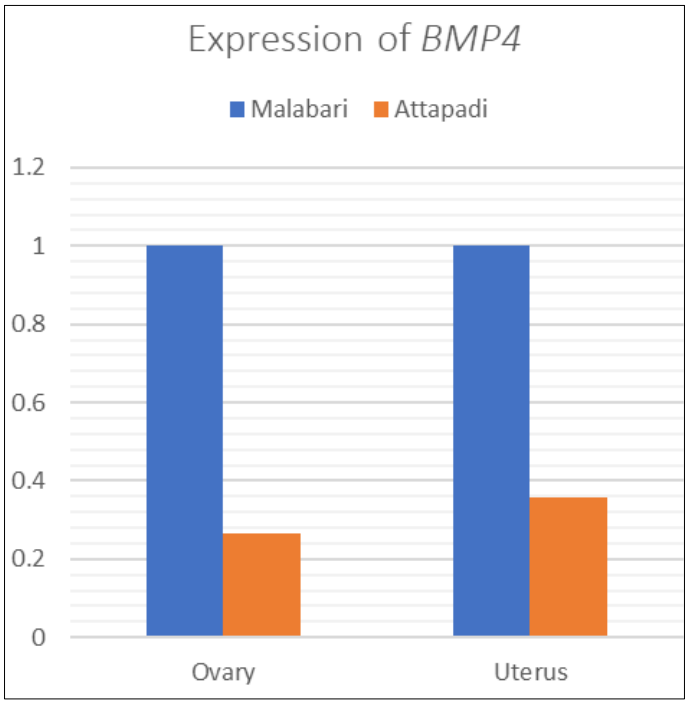

Fig 7: BMP4 mRNA expression levels in ovary and uterus of Malabari and Attappady Black goats

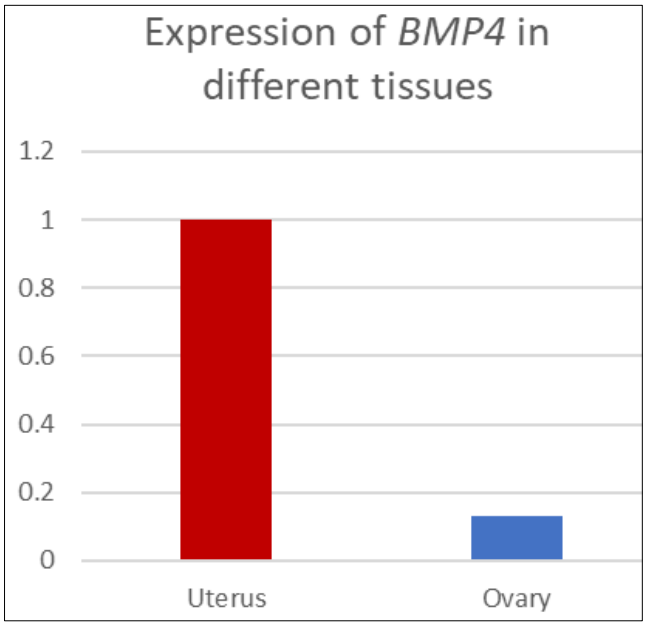

Fig 8: BMP4 mRNA expression levels in uterus and ovary

In the current study a significant difference was observed in the BMP4 gene expression in the ovarian tissues of Malabari goats compared to Attappady Black. This could be due to the higher prolificacy of Malabari compared to the Attappady Black breed. Previous studies have indicated the presence of BMP4 mRNA in different layers of the ovary [5] (Fatehi *et al*., 2005). Tanwar and McFarlane (2011) reported the presence of BMP4 protein during the various stages of ovarian folliculogenesis [16]. Additionally, the BMP4 gene plays a role in skeletal development during embryogenesis which could justify the high mRNA expression in the uterus tissue.

4. Conclusion

A significant difference in the expression of BMP4 gene was observed between the ovaries and uterine tissues of the two breeds. Keeping the uterus as control, tissue-wise comparison revealed that mRNA levels of the BMP4 gene in the uterus and ovary differed significantly. The ovary of the Attapady Black goat breed had a 0.26-fold lower expression of BMP4 than that in the ovary of the Malabari goat breed. The present study could conclude that a significant difference was observed in the expression profile of BMP4 gene between tissue samples as well as between the tissue samples of the two breeds.

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