#### **Original Paper**

# The morphological changes of uterus in postnatal development of heifers

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The aim of this work was to describe the microscopic and submicroscopic changes of the uterus of 20 Pinzgau heifers in their postnatal development (12<sup>th</sup>, 24<sup>th</sup> and 36<sup>th</sup> week of age) and in the experimental form of subclinical hypoglycaemia. Uterine specimens were obtained from the uterine horns by a vivisection for histological studies. Samples were fixed for light microscopy (LM) in formal dehyde and for scanning by electron microscope (SEM) and transmissive electron microscopy (TEM) in glutaral dehyde. Subsequently, the samples were processed in the usual manner as used in the LM and electron microscopic studies laboratories. The uterus increased to  $24^{\text{th}}$  weeks by 19.4%. The uterine weight decreased significantly (P < 0.01) at the time of hypoglycemia (-14.6%). When the ovarian weight increased (+48.8%) to 36<sup>th</sup> week of age, the uterine weight increased by 39.9%. At the time of hypoglycaemia, the ovarian weight decreased by 4.4% and the uterine weight decreased by 17.1%. Endometrial development was mostly pronounced between  $12^{\text{th}}$  and  $24^{\text{th}}$  week of age (P < 0.05). In particular, the superficial (+24.8%) and glandular epithelium (+25.9%) developed. Slower development continues up to 36<sup>th</sup> week, but in animals with hypoglycaemic development it stagnates (-4.1% and -18.8% respectively). The nucleo-cytoplasmic ratio was gradually reduced in luminal epithelial cells (N : C 1 : 3.2 to 1 : 2.9 respectively) and narrowed in glandular epithelial cells (N : C 1 : 2.3 to 1 : 1.7 respectively). A larger decrease was observed in the glandular epithelium. Mitochondria (M) increased the volume in both epithelium types (22.4% and 28.2%). In a hypoglycaemia is volume of M low (-18.4% and -16.2%). The rough endoplasmic reticulum (rER) increased in volume (+6.5% and +5.6% respectively) in both types of epithelial cells. Hypoglycaemia has been shown to decrease the volume of rER approximately equally (-10.9% and -10.2%). Macroscopic, microscopic and submicroscopic cell changes of endometrium are described in postnatal development and experimental subclinical hypoglycemia of heifers. There is a clear manifestation of the energy deficit in the retardation of growth and developmental changes.

Keywords: heifers, uterus, postnatal development, histology, hypoglycaemia

### 1 Introduction

The uterus of the mammals is the organ of pregnancy (Senger, 2011). It is a two-armed, muscular organ consisting of cervix, corpus and two cornua. The body of the uterus of a cow is short. Bielaňski (1972) reports a length of 9–12 cm, but Salisbury et al. (1985) only 2–5 cm. The body is formed from the caudal parts of the corners which form the longest part of the uterus, 25-30 cm (Bielaňski, 1972; Salisbury et al., 1985), but according to McDonald (1975) it is 35-40 cm. The wall is composed of the mucosa (endometrium), the muscular layer (myometrium) and the entire uterus is covered by serosus (perimetrium). The size of the uterus varies depending on the breed, age, birth rate, pregnancy, and, if appropriate, its health. The cervix is a sphincter, has a thick wall and a narrow canal. The cervix creates a barrier between the uterus and the external environment. The length of the neck is from 1.5 cm in heifers to 8 cm in cows. According

to Bielaňski (1972), heifers have 6.6 cm long necks and cows 5–11 cm long and according to McDonald (2003) 8–10 cm in multi-breeding cows. The length and diameter of the cervix is greater for non-pregnant adult cows than for heifers. Most non-pregnant exotic cattle have a 7–10 cm long cervix (Ali et al., 2003), but Bello et al. (2012) found cervix of 8.0 cm with the same diameter. The mucous membrane of the cervix forms transverse folds. According to Salisbury et al. (1985) is the uterus 24–40 cm long. The postnatal development of the reproductive organs is continuously linked to the prenatal development and can continue even after puberty until sexual maturity. Intense development up to the 6<sup>th</sup> month of age also indicates an increase in uterus weight, which is related to the level of gonadal hormones. By the 6<sup>th</sup>-8<sup>th</sup> month of age it is relatively stable and, according to Foote (1972), consistent with physical development. The results of the Desjardins and

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Hafs studies (1969) confirm that the weight of the uterus is multiplied 22.5 times by the 10<sup>th</sup> month of age, but its enlargement continues beyond that time. In contrast, heifer multiplies its body weight by only 7.8 times. With increased endocrine ovarian activity associated with the onset of the estral cycle (40<sup>th</sup> week of age), a marked uterine development is seen (Foote, 1972; Williams and Amstalden, 2010). Desjardins and Hafs (1969) describe the slow, linear ovulation of the uterine cervix to the first ovulation. Similarly, the growth of the cervix and vagina is slow until the 4<sup>th</sup> month of age but followed by rapid growth after the first ovulation. It correlates with the growth of ovaries until the 5<sup>th</sup> month of age when their growth is stabilized. Honaramooz et al. (2004) describes intense postnatal development of the uterus of heifers, excluding the two-week period, up to the age of 6 months, followed by a tranquilization phase and re-intensive growth from the 8th to the 15th month of age. The reason for the two-phase development of the ovaries and the tubular reproductive tract is not entirely clear. This suggests that increased gonadotropin secretion (especially Luteinizing Hormone - LH) in the early postnatal period of heifers may reflect maturing changes in the regulation of gonadotrophin secretion prior to an estrogen suppression (Evans et al., 1994). Subsequent reduction of LH secretion by negative feedback and further enhancement of a follicular estrogen capacity can prevent and predict pubertal ovulation of heifers (Day et al., 1984, 1987). Bartol et al. (1995) studied the effect of progesterone (P) and estradiol (E) implants in neonatal heifers and changes in their adult endometrium. Regardless of the age at the time of application, P and E neonatal exposure caused a reduction in uterocervical weight by 35%, myometrial reduction by 23%, and endometrium by 27% compared to untreated animals. The endometrial gland density was reduced by 40%. This effect depends on the age in which the implant was administered. Endometrial gland density was reduced by 65% in case of treatment at birth, 22%, and 33% in case of treatment on day 21 or 45 after birth.

### 2 Material and methods

## 2.1 Animals

Twenty Pinzgau bred heifers of 4 weeks of age, weighing 52.4 kg, were selected for the experiment. Diet was permanently optimized for 15 animals. In 5 animals was at 32 weeks of age (body weight 148.9 kg) induced the subclinical hypoglycaemia (glucose in blood plasma achieved – 1.9–2.2 mmol, – using commercial spectrophotometric kits, plasma was analyzed for glucose – Sigma Tech, Bull.; Sigma Chemical, St. Louis). This condition lasted until the 60<sup>th</sup> week of age

(241.3  $\pm$ 3.1 kg). Average daily gain was 447.0 g. Heifers with the hypoglycaemia were at this time slaughtered too. 15 heifers were divided by 5 animals into 3 groups according to the age at which they were killed (12, 24 and 36 weeks). The animals were periodically weighed and their metabolic and health status checked.

## 2.2 Sampling and samples processing

All animals were slaughtered in line with the current state by usual method and the reproductive organs were removed immediately after draining of blood. The samples from uterus were taken from endometrium for light (LM), transmission (TEM) and scanning electron microscopy (SEM). Samples for LM were fixed in 10% formol, dehydrated by sequence of alcohols and deluged into paraffin. 8–10 µm thick slices were made from blocks, they were coloured by haemalauneeosine and by greens trichrome. For histochemical proof of glycogen and PAS - positive substances were used samples fixed in Gendres solution (Vacek, 1974), with PAS reaction (Schiff's reaction periodic acid - Schiff). Sections were evaluated with LM (Olympus Provis AX) with a program bound for assessment of individual morphological structures - Statgraphics ver. 7, Image ProPlus (Spectra Services Inc, NY) and MS Excel 2000. Samples from the same parts and places of oviduct were taken for electronmicroscopic studies (TEM, SEM). They were fixed in 4% solution of glutaraldehyde paraformaldehyde (pH 7.4) with 0.08 M – cacodylate buffer (pH 6.9–7.1). We used 1% osmiumoxid with phosphate buffer (Milloning, 1962) for post fixation for TEM, samples were rinsed by Milloning's phosphate buffer and sucrose. They were dehydrated by ascending sequence of ethanols, rinsed by propylene oxide and deluged in the compound Durcupan ACM (Fluka). Semi-thick (1 μm) and ultra-thin slices were made on ultramicrotome (LKB 8800 III). Semithick slices were coloured by Toluidine blue and assessed (Olympus Provis AX). Samples for SEM were rinsed and dehydrated in ascending sequence of acetones and desiccated with the help of CO<sub>2</sub> (CPD Polaron, England) after fixation (3 hours). On the fixtures, the dry samples were then metalized with 20 nm thick layer of gold by vacuum steaming. Ultra-thin slices were contrasted with lead citrate (Reynolds, 1963) and uranyl acetate. Electronograms were made with TEM (TESLA BS 500) and SEM (TESLA BS 301).

Morphometric methods were used for objectification of results (Weibel et al., 1966; Mráz and Polónyi, 1988).

## 3 Results and discussion

According to Mukasa and Mugerwa (1989) is the uterus a muscular organ consisting of a body, about 4 to 5 cm long, and two uterine horns, each 15 to 25 cm in length and 1 to 3 cm in diameter. The length of the cervix



Figure 1 Comparison of the uterus weight (g) and the body weight (kg) of the heifers at different ages and with hypoglycaemia

varies from 1.5 cm in heifers to 8 cm in multiparous cows of larger breeds. With the exception of the cervix lengths, it is not interpreted whether these attributes are from the cow uterus or from the heifer uterus. Age of the animals is absent too. Similarly, others (Salisbury et al., 1985; McDonald, 1975; Ali et al., 2003; Bello et al., 2012) do not report the ages of the animals. According to our results it is probably about cyclic heifers or cows, because these attributes are near to 36 wks and 60 wks old animals (Table1). The smallest changes of a development were on the body of the uterus. In other parts were obvious changes (P < 0.05, resp. P < 0.01). After rapid growth of the uterus till 24<sup>th</sup> week of life, stagnation occurs in the next period. The uterus increased to 24th week by 19.4% but till 36<sup>th</sup> week by only 3.5%. There was small correlation (r 0.135) between the length and weight of the uteri and the body weight. The weight of the uterus has dropped significantly (P < 0.01) at the time of hypoglycaemia (-14.6%, Figure 1). Honaramooz et al. (2004) describes the intensive postnatal development of the uterus of heifers, with the exception of the two-week period, up to 6<sup>th</sup> month of age, followed by a resting phase and a recurrent growth from 32<sup>nd</sup> to 60<sup>th</sup> month of age. The reason for the biphasic nature of ovarian and tubular reproductive tract development remains unclear. It has been suggested that increased



gonadotropin secretion (particularly LH) in the early post-natal heifer, may reflect maturational changes in the regulation of gonadotropin secretion prior to suppression by estrogen (Evans et al., 1994). The weight of the uterus was much more affected by changes in ovaries (r 0.995) of the first 36 weeks, as well as at the time of hypoglycaemia (Figure 2). An increase in weight of ovaries +48.8% to 36<sup>th</sup> week of age, the weight uterus increased +39.9%. At the time of hypoglycaemia, the weight of the ovaries decreased -4.4% and the uterine weight decreased -17.1%. This confirms the significant influence of ovaries on the changes occurring in the uterus during its development and the lesser dependence on changes in body weight. Administration of estrogens to neonatal gilts from birth affects uterine growth and endometrial development acutely at both structural and biochemical levels (Yan et al., 2008). Treatment with estradiol valerate 2 weeks after birth increased uterus wet weight and advanced endometrial development to post-natal day 14 as reflected by increased glandularity and premature development of endometrial folds (Tarleton et al., 1999).

This fact also supports the trend in terms of growing follicles ( $\pm 8$  mm), which has a tendency to increase number of follicles and to  $36^{th}$  week of age of heifers. This is also the same, declining trend at the time of hypoglycaemia in both (Figure 3). Foote (1972) and

Ages/wks	Cervix (mm)	Body (mm)	Horn (mm)	Uterus (mm)	Uterus weight (g)	Body weight (kg)
	x±s	x ±s	<i>x</i> ±s	x ±s	x±s	x±s
12 wks	29.3ª ±0.05	29.4 ±0.41	69.7ª ±0.47	128.1 <sup>b</sup> ±1.12	61.4 <sup>b</sup> ±22.0	94.1 <sup>b</sup> ±7.22
24 wks	35.0 ±0.12	30.3 ±0.33	93.9 ±0.29	159.0 ±1.51	135.1 ±31.0	157.8 <sup>b</sup> ±11.3
36 wks	35.8 ±0.15	30.1 ±0.10	96.4 ±0.35	164.7 ±1.99	153.6ª ±10.0	221.7 ±10.1
60 wksH	36.6 ±0.326	30.7 ± 0.46	97.1 ± 0.96	164.4 ±2.86	131.1 ±18.0	241.3 ±3.1

**Table 1**The length and weight of the uterus and the body weight of heifers (No5/group)

<sup>a</sup> P <0.05; <sup>b</sup> P <0.01; H – hypoglycaemia



**Figure 3** Number of follicles (± 8mm) and weight of the uterus (g) in different age of heifers



Figure 4 The dependence of the heigth of endometrial epithelium on the number of ovarian follicles SE – superficial epithelium, GE – gladular epithelium, wks – weeks, H – hypoglycaemia





Part of the 24-weeks old heifer endometrium E – luminous epithelium, L – lumen, LP – lamina propria, BV – blood vessel, arrow basal membrane; Toluidine blue,  $\times$ 1,260



Figure 7 Uterine gland of heifer at 12 weeks of age E – epithelial gland, L – lumen, BV – blood vessel, LP – lamina propria, arrow basal membrane; Toluidine blue, ×1,260



Figure 5

Part of the 12-weeks old heifer endometrium LE – luminous epithelium, L – lumen, LP – lamina propria, BV – blood vessel, arrow basal membrane;, Toluidine blue, ×1,260

Williams and Amstalden (2010) confirms that marked increase in uterine development is seen in increasing ovarian endocrine activity associated with the onset of estral cycle (40<sup>th</sup> week of age). These data suggest that



Figure 8 Uterine E – epith arrow wi

Uterine gland of heifer at 24 weeks of age E – epithelial gland, L – lumen, LP – lamina propria, arrow with BM basal membrane; Toluidine blue, ×1,260

uteri of heifers are influenced by significant levels of gonadal hormones during the seventh month of age before the onset of first estrus. These reflections are in agreement with similar investigation for rats (Desjardins



Figure 9 The epithelial cells of lumen (24 wks of age), they have the microvilli at the apical end MV – microvili, Gc – glycocalyx, EDV – electron densite vesicles, JC – juctional complex; TEM, ×14,600

et al., 1968). Development of the uterus involves a series of morphogenetic and cytodifferentiative events that establish the framework for tissue function in adulthood. In the pig, uterine glands are absent at birth (postnatal day 0) and the uterus is estrogen receptor- $\alpha$  negative (Tarleton et al., 1998; Yan et al., 2006). However, during the first 2 weeks of life, uterine glands differentiation is evident in both nascent glandular epithelium and endometrial stroma (Tarleton et al., 1999). Post-natal development of the uteri before puberty can also be seen in changes in endometrial structures. Endometrial development was most pronounced between 3rd and  $6^{th}$  month of age (P < 0.05). In particular, the superficial (+24.8%) and glandular epithelium (+25.9%) developed in contrast to interstitial tissue (Table 2). Slower development continues till 9th month, but in animals with hypoglycaemia the development stagnates and in the 15<sup>th</sup> month of age (Figure 13, 14), the superficial and glandular epithelium has a volume of approximately six month heifers (-4.1%, resp. -18.8%). Chelikani et al. (2003)



Figure 10 The cells of the luminal epitelium (36 wks old), they have on the apical ends well structural villi (arrows) and cells contain electrodensite vesicles (EDV) JC – juctional complex, TEM, ×14,000

reported that the development of follicles depends only on age and not on the energy value of the feed. Changes in epithelial volume were caused by a change in epithelial cell height, probably due to an increase in the number of larger follicles (±8.0 mm) as documented in Figure 4 and 6. The luminal uterine epithelium of 12 weeks old heifer is unlike the epithelium at the age of 24 weeks, it is low and irregularly layered (Figure 5). A similar picture to see even in the glandular epithelium, which is highly heterogeneous (Figure 7, 8). The cells of the epithelium of the glands are not very dense, "quasi empty ". The cells of the superficial epithelium at the age of 24 weeks already have the apical end of microvilli, which were not available before.

Age/wks	Superficial epitelium	Glandular epitelium	Glandular lumen	Interstitium	
	x ±s	x ±s	x ±s	x ±s	
12 wks	1.73 ±0.62	4.01 ±0.67	0.97 ±0.42	93.30 ±2.37	
24 wks	2.30ª ±0.66	5.41ª ±0.41	1.19 ±0.51	91.10 ±3.43	
36 wks	2.41 ±0.59	5.97 ±0.74	1.17 ±0.46	90.45 ±4.21	
60 wksH	2.31 ±0.54	4.85 ±0.61	1.20 ±0.44	91.34 ±3.52	

 Table 2
 The relative volume of the endometrial components (% from mucous)

<sup>a</sup> 12:24; P < 0.05



Figure 11 Ciliations of the endometrial cells of 36-wks old heifers, SEM, covered by gold, ×16,200



Figure 12 The nucleo-cytoplasmatic ratio of the endometrial epithelium N = 1; GE – glandular epitelium; SU – superficial epitelium; N – nucleus; C – cytoplasm

Endometrial cell ciliations (Figure 10, 11) are usually associated with the proestral and estral stages of the animal cycle (Wick and Kress, 2002, Akinloye and Oke, 2014). Postnatal development and energy deficit also marked the internal structure of the cells. Although the closest nucleo cytoplasmic (N : C) ratio in immature



Figure 13 Uteri luminal epithelium of heifers with subclinic hypoglycaemia is heterogeneous, without ciliation L – lumen, E – epithelium, LP – lamina propria, BV – blood vessel) Toluidine blue, ×1,260

and young cells is described and later relatively stable, in luminal epithelial cells and glandular epithelial cells, this ratio gradually narrowed down (Figure 9). A larger decrease was observed in the glandular epithelium (Figure 12). The lowest values can be seen in animals with hypoglycaemia.

Postnatal development of the uterus was also reflected in ultra-structural changes in epithelial cells of the luminal and glandular epithelium. Mitochondria (M) increased the volume in both epithelium types (22.4% or 28.2% respectively), (Table 3). However, over a 24-week period of hypoglycaemia, the volume M fell approximately equally (18.4% and 16.2% respectively). A much more stable value preserved the rough endoplasmic reticulum (rER). Both types of epithelial cells increased the volume in the cytoplasm (+6.5% and +5.6% respectively). The negative effect of hypoglycaemia has been shown to decrease the volume of rER approximately as much in the luminal as in the glandular epithelium (-10.9% and -10.2% respectively). The occurrence of lysosomes (L) has been unstable and it is difficult to evaluate changes in volume.

**Table 3**Volume of the organelles of the endometrial epithelium (% of the cytoplasm)

Age/wks	Superficial epiteli	um		Glandular epitelium			
	x ±s			x±s			
	М	rER	L	М	rER	L	
12 wks	9.7 ±4.2	8.6 ±3.7	3.03 ±0.1	8.4 ±2.2	8.3 ±3.9	6.1 ±0.2	
24 wks	10.3 ±4.7	8.4 ±2.6	8.4 ±0.07	9.6 ±2.9	8.8 ±2.8	4.09 ±0.4	
36 wks	12.5 ±6.4	9.2 ±3.4	7.04 ±0.04	11.7 ±4.4	8.8 ±3.1	6.3 ±0.2	
60 wksH	10.2 ±5.3	8.2 ±4.1	9.7 ±0.2	9.8 ±4.6	7.9 ±3.7	6.6 ±0.9	

M - mitochondria; rER - rough endoplasmatic reticulum; L - lysosome



Figure 14 Epithelial cells are little denseness and they contain many vesicles, we see starting of karyolysis N – nucleus, V – vesicles, M – mitochondria, JC – juctional complex, arrows collapse of karyolema), TEM, ×29.500

### 4 Conclusions

This work describes structural quantitative and qualitative changes of heifer uterus during their postnatal development until the pubertal period (from 12<sup>th</sup> wk to 36<sup>th</sup> wk). Changes in the heifer uterus structure with inducted subclinical experimental hypoglycaemia from 36<sup>th</sup> wk of age up to 60<sup>th</sup> wk were also assessed. Progressive development changes in mucous structures, especially epithelium mucous component were confirmed. We discovered different development and quantitative changes in superficial and glandular cells which are under steroid pre-pubertal control. Based on the changes in hypoglycaemic animals we can assume the importance of sufficient energy subsidising during development, because these animals based on comparison with other observed animals and despite their age of 60 wks (reproduction age) they had the development of uterus structures on the level of animals in pre-pubertal age.

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