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IN VITRO PROGESTERONE METABOLISM BY LIVER CELLS : DETECTION AND IDENTIFICATION OF METABOLITE

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ABSTRACT : The purpose of this study was to evaluate the dose dependent (0, 5and 10µg/ml of progesterone) *in vitro* secretion and conversion of progesterone metabolite in cultured liver cell of water buffaloes. Buffalo Liver samples from fetus of approximately 50-60 days old were collected from a local slaughterhouse. The samples were then processed and cultured in (serum containing) appropriate cell culture medium and incubated separately with (progesterone) the previously mentioned two dose combinations. At the end of the 12 hrs of incubation period, the progesterone metabolite were extracted from liver cell culture supernatant and detected using high performance liquid chromatography with a reversed-phase (HPLC). Comparison of the results of the two treatment depicted 5µg/ml progesterone as the most potent conversions into its metabolite in bubaline cultured liver cells. In conclusion, secretion and conversion of progesterone into metabolites data clearly indicate for the first time that liver play an important role in metabolism of progesterone in buffalo that are then secreted in the bile. The highest conversion of progesterone metabolite with 5µg/ml of progesterone concentration might be associated with the liver function. The principal progesterone metabolite by HPLC was 5β-pregnane-3β (52.37%) and less 5-Prgnane 3β (24.53%), with 5µg/ml of progesterone concentration during 12hrs of incubation. These results are compared with studies in other ruminants in which pregnanes in the 5β-configuration and 5α-configuration are the dominant form in the faeces.

Key words : HPLC, progesterone metabolites, liver cells, buffalo fetus.

INTRODUCTION

The amount of any hormone in circulation is regulated by the rate of hormone release from the endocrine gland and the rate of hormone degradation in the organs that metabolize it (Jorge *et al*, 999). Previous reports suggested that the uterus and ovaries are not essential for the reduction of progesterone in men (Hamblen *et al*, 1940) and in hysterectomized women (Venning and Browne, 1940; Jones and TeLinde, 1941) rise in urinary pregnanediol, a metabolite of progesterone has been seen in urine who were injected with progesterone. *In vitro*, labeled progesterone were metabolized by uterine tissue of non-pregnant rats to several steroids, the most abundant of which was 3α- hydroxy-4-pregnen-20-one (Lisboa and Holtermann, 1976). The endometrium does metabolize progesterone metabolism during pregnancy in cow (Eley *et al*, 1983), guinea pigs (Glasier *et al*, 1994) and in endometrial epithelial in bovine (Jamshidi *et al*, 2007). In human progesterone metabolites play a role in parturition (Sheehan *et al*, 2005; Sheehan, 2006). However, the liver appears to be important in metabolized the hormone. Schwarzenberger *et al* (1996) tentatively identified progesterone metabolite (5α-pregnane-3, 20-dione, 5 α-pregnane- 3α-ol-20-one and 5α-pregnane-3β-ol-20-one) in black rhinoceros. Whereas, Heistermann *et al* (1998) recognized principle progesterone metabolite (5β-pregnane-3α, 20α-diol, 5β-pregnane-3α-ol-20-one and a second pregnanediol) in southern white rhinoceros by HPLC. Tripp *et al* (2009) detection progesterone metabolite (3α-hydroxy-5α-pregnan-20-one and 5α-pregnane-3, 20-dione) in plasma of female Florida manatee (*Trichechus manatus latirostris*) by GC/MS/MS.

In African and Asian elephants, the 5á-reduced progestins were predominant, including 5á-pregnane-3, 20-dione (5α-DHP) and 3α-hydroxy-5α-pregnan-20-one (5á-P3-OH) (Hodges *et al*, 1997). 5α-DHP is predominant in the rock hyrax (Kirkman *et al,* 2001). In African and Asian elephants, 5á-DHP predominates (Hodges, 1998) and in mare (Holtan *et al*, 1991) during pregnancy. 20α hydroxy- 4-pregnen-3-one (20á-hydroxyprogesterone; 20α-OHP) a progesterone metabolite, detected in rat and bovine (Lu and Judd, 1982; Tsumagari *et al,* 1994). The liver is a major site of hormone metabolism and it contributes to the maintenance of peripheral concentrations by degrading most hormones. Therefore, in the present study, we examined the dose dependent effects of progesterone on progesterone metabolites conversion in the cultured bubaline liver cells.

MATERIALS AND METHODS

Isolation and culture of liver cells

For isolation of liver cells, buffalo fetus of approximately 50-60 days old were taken from gravid uterus, collected from nearby slaughter house. The gravid uterus was incised to collect the fetus in a beaker containing sterile (1x) PBS. Cut the fetus and take liver tissue under laminar air flow. The fetal liver tissue was chopped in a plate containing Dulbecos Modified Eagle Media (DMEM) supplemented with fetal bovine serum (15%) and antibiotics (Streptopenicillin) @10000 units/ml. The cells were separated by in-out pipetting and the cell suspension was centrifuged at 500 rpm for 2-3 min. The supernatant containing single cells were collected in a separate centrifuge tube. This tube was centrifuged at 1000 rpm for 5-10 min and the supernatant was discarded. The washing was repeated at least three times. The cells were then resuspended in medium (DMEM + FBS+ Leukemia inhibitory factor,10 ng/ml and antibiotic) and further cultured in tissue culture plate in a CO_2 incubator at 37°C, 5% CO_2 and maximum humidity. Once the cells achieved 70-80% confluency, they were continuously propagated by trypsinisation method in the same medium supplemented with (15%) FBS. After it Progesterone was incubated in culture media in different concentration like 0, 5µg, 10 µg/ml culture volume. Culture supernatant was taken after 12hrs of incubation. Culture supernatant was extracted with diethyl ether (Heistermann *et al*, 1998), briefly 0.3ml of culture supernatant and 5 ml diethyl ether mix and vortexed for 10 min after that it was kept for 1 hr on ice then supernatant was collected, dried and reconstituted in 1ml of methanol, then vortexed for 1min and preserved in deep freezer until HPLC analysis.

High performance liquid chromatography (HPLC) of culture supernatant

Pure sample of Progesterone and its metabolites like 21-Hydroxy progesterone, 5α- Pregnane 3α, 5β-Pregnane-3â and 5-Prgnane-3â were obtained from Steraloids Inc P.O. Box 689 Newport, RI 02840. All standards were of analytical grade. HPLC grade acetonitrile and water were solvent from J.T Baker, USA. Mobile phases used in this study were filtered through 0.22µm nylon membrane filters (from Millipore, Ireland) and using vacuum filtration assembly and was sonicated using a bath type ultrasonicator before HPLC.

Procedure

Reversed-phase HPLC analysis was performed and separation was achieved with Acetonitril : water (30:70 vol/vol) gradient solvent system. Gradient programme: 0.0 to 2.0 min (70% water phase and 30% acetonitrile) 2.0 to 3.0 min (50% water phase and 50% acetonitrile) 3.0 to 5.0 min (10% water phase and 90% acetonitrile) 5.0 to 9.0 min (70% water and 30% acetonitrile phase) and 9.0 to 20.0 min (70% water and 30% acetonitrile phase). The volume of sample injected was 20 ìl. The HPLC system (Waters Miliford, MA, USA), which consists of Waters-515 binary pump, Rheodyne injector-7725 with 20 µl loop, waters column C-18 (250 x 4 mm, dp: 5 µm) and Waters UV detector-2487 at 254 nm. The pooled extracted liver cell culture supernatant was analyzed successfully.

RESULTS AND DISCUSSION

Primary isolation and expansion of Buffalo liver cells

The results of isolation, culture and propagation of buffalo liver cells have been presented n Fig 1. The results indicated that the cells isolated from buffalo liver started attachment after 3-4 days and formed few small colonies as monolayer on day 6-7 of culture. These cells took about 8-10 days to make 70-80% confluence. The cells grown in the present study appeared morphologically homogenous and maintained similar morphology during different passages. These cells propagated after every 7-8 days. Fig. 1 showed the liver cells from buffalo fetal at passages 0, 1and 2.

The effects of progesterone on buffalo liver homogenate

The results of incubating progesterone concentration (0, 5 and 10 µg/ml) with buffalo liver preparations under a variety of conditions are illustrated. It may be seen that progesterone is metabolized by Buffalo liver.

Detection and identification progesterone metabolites by HPLC

To determine whether buffalo liver cells could metabolize progesterone or not, the cells were incubated with 0, 5 and 10µg/ml of P4 concentration and the metabolites in the culture supernatant subsequently separated by HPLC. The results showed in table 1 and figs. 2, 3 that progesterone was metabolized at the rate of 76.91% and 53.52% respectively with 5 and 10µg/ml of P4 concentration by liver cells during 12hrs of incubation. This separation of progesterone metabolite from culture supernatant was separated on a reversed-phase ACCLAIM C18 (250X4.6), 5 MICRON column with a solvent system of acetonitril (ACN)/water (H_2O) and a flow rate of 1ml/min. This system was used initially because it gave a good separation of progesterone. To determine if these metabolites were conjugated steroids, they were separated into conjugated and free forms prior to separation by HPLC.

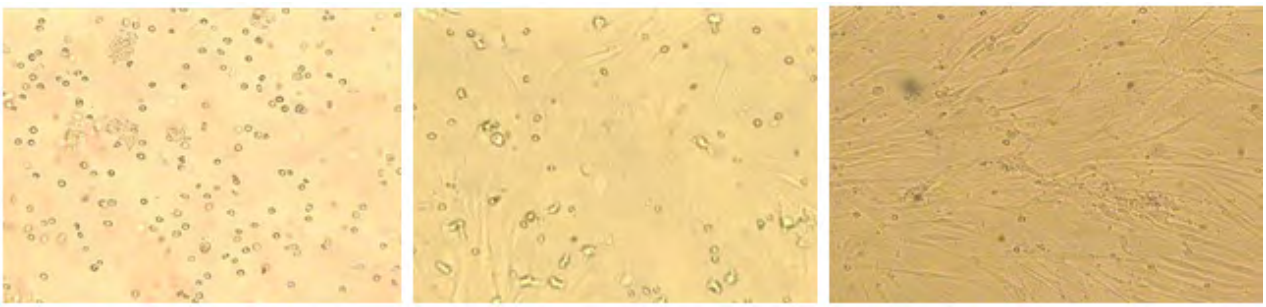
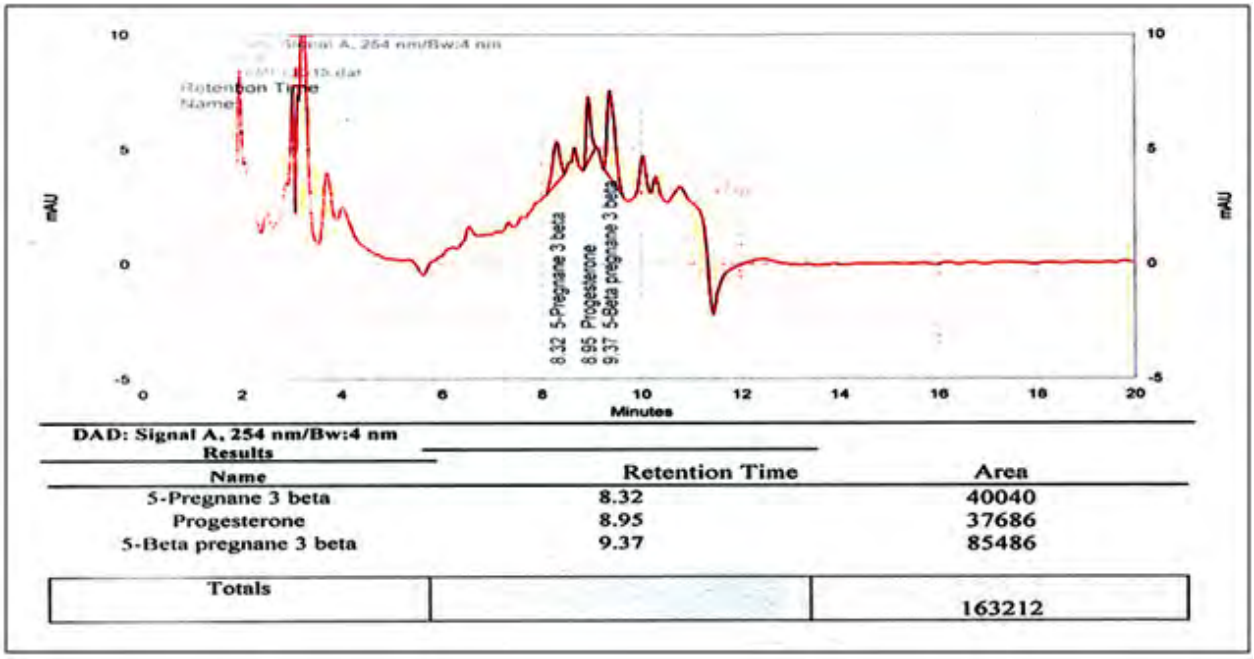


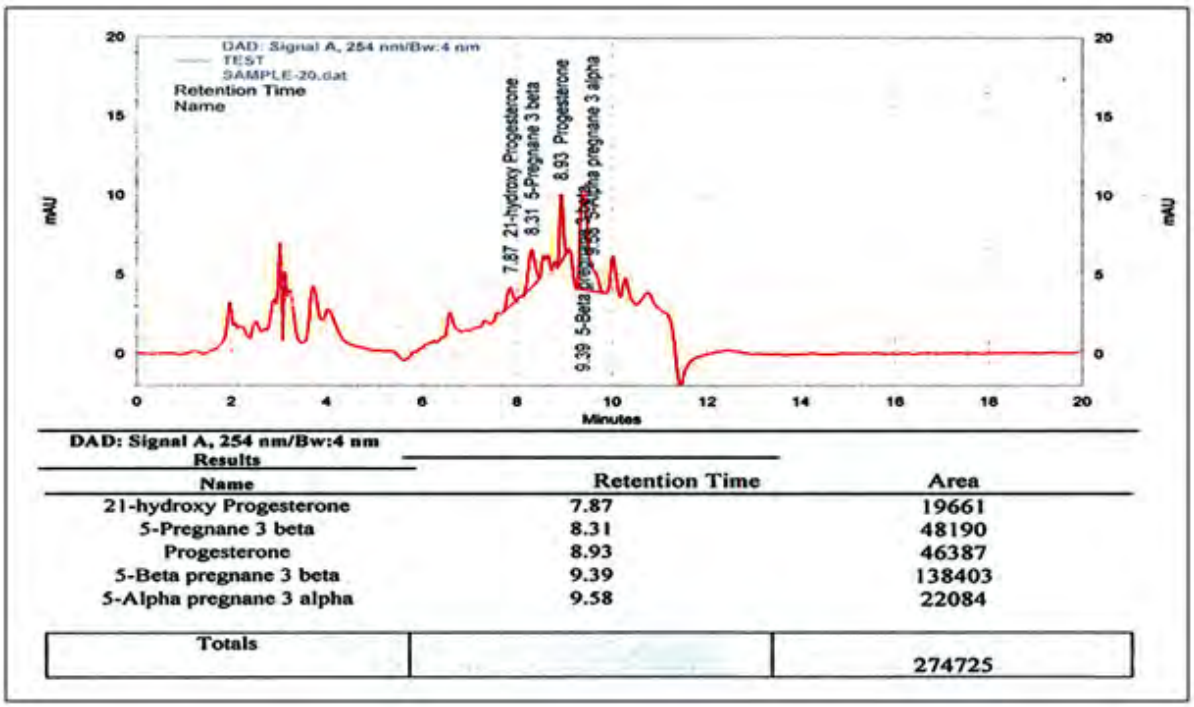
Fig. 1 : Showed the liver cells from buffalo fetus at passages 0, 1 and 2.



DAD: Signal A, 254 nm/Bw:4 nm
Results

Name	Retention Time	Area
5-Pregnane 3 beta	8.32	40040
Progesterone	8.95	37686
5-Beta pregnane 3 beta	9.37	85486
Totals		163212

Fig. 2 : Chromatogram of 5µg/ml progesterone incubated in culture media, extracted after 12hrs incubation.



DAD: Signal A, 254 nm/Bw:4 nm
Results

Name	Retention Time	Area
21-hydroxy Progesterone	7.87	19661
5-Pregnane 3 beta	8.31	48190
Progesterone	8.93	46387
5-Beta pregnane 3 beta	9.39	138403
5-Alpha pregnane 3 alpha	9.58	22084
Totals		274725

Fig. 3 : Chromatogram of 10µg progesterone incubated in culture media, extracted after 12hrs incubation.

Table 1 : Mean percent area of progesterone metabolite in liver cell culture supernatant.

Group	21-OH-Progesterone (% area)	5-Prgnane 3â (% area)	5â-Pregnane-3â (% area)	5á -Pregnane-3á (% area)	Unmetabolized Progesterone (%)	Total conversion of P4 (%)
Control	0.00	0.00	0.00	0.00	0.00	0.00
5µg	0.00±0.00	24.53±0.00	52.37±0.00	0.00±0.00	23.09±0.00	**76.91**
10µg	7.15±0.00	11.33±6.21	31.46±18.91	7.12±0.91	46.48±29.61	**53.52**

Liver cell culture supernatant analyzed by HPLC resulted in identification of several P4 metabolites. The HPLC did not detect any additional progestins other than those for which standards were included. We hypothesized that P4 metabolites in the liver cell culture could be identified using HPLC, which we confirmed. The results showed in table no.1 and fig no. 2 that Progesterone was converted (76.91%) into 5-Prgnane 3β (24.53%) and 5β-Pregnane-3β(52.47%) with 5µg/ml of P4 concentration during 12hrs of incubation similar result was also given by Cooke and Taylor, (1962) found that 72.5% of progesterone metabolite with 4µg/ml by female rat- liver homogenate. Although result showed in table 1 and fig 3 that progesterone converted (83.12%) into 21-OH-progesterone (7.15%), 5α-prgnane 3α (8.03%), 5-prgnane 3β (17.54%) and 5β-pregnane-3β (50.37%) respectively with 10µg/ml of progesterone concentration during 12hr incubation, similar finding was observed by Taylor (1955) in rabbit liver homogenate with 10µg/ml instead of buffalo liver and he observed metabolite in 5α-pregnane-3:20-dione, 3α -hydroxy-5α -pregnan-20-one and unable to isolate any steroid of the 5β -pregnane series. The results of present study indicate that 21-OH-Progesterone metabolite produced after 12 hrs of incubation, whereas 20α-reduced progestins were the progesterone metabolites produced by human cyclic endometrium (Sweat and Bryson, 1969).

In equines, elephants, and hyraxes, 5α-DHP (Dihydroxy progesterone) is biologically active metabolite in serum, due to its high binding affinity for that species' endometrial progestin receptors (Meyer *et al*, 1997; Hodges *et al*, 1997; Jewgenow and Meyer, 1998 and Kirkman *et al*, 2001). In the horse, 5α-DHP receptor affinity is greater than that of P4 itself (Ousey *et al*, 2003). In the diestrus rat, the concentration of 20α-OHP is four- to sixfold greater than that of P4. Bovine cotyledons also contain 20α- OHP, which increases and peaks in late gestation (Months 7 to 8) and subsides after parturition (Month 10) (Tsumagari *et al*, 1994).

Lance *et al* (2001) identified a total of 33 pregnanes in the $C_{21}O_2$ series from fecal extracts of a pregnant black rhinoceros (*Diceros bicornis minor*), 81% of the pregnanes by mass were in the 5á-configuration. Heistermann *et al* (1998) study in female Sumatran rhinoceros in which ^{14}C-progesterone was injected, no radiolabeled progesterone was recovered in the feces, but more than 99% of injected radioactivity was accounted for in the fecal fraction and only trace amounts in the urine. The principal route of excretion of progesterone metabolites in most mammals studied is via the feces (Palme *et al*, 1996).

In rats and humans, and presumably other mammalian species, the passage of ovarian venous blood through the liver reduces the double bond between carbons 4 and 5 in the progesterone molecule to 5α (allopregnane) or 5β (pregnane) compounds that are then secreted into the bile (Taylor, 1971; Adlercreutz and Martin, 1980; MacDonald *et al*, 1983; Palme *et al*, 1996). In the gut the steroids are further modified. Hydroxylases for all positions in the progesterone molecule are known to occur in vertebrates except for C-4, 5, 8, 9, 10, 12 and 13. Micro-organisms have been identified, however, that can hydroxylate steroids not only in the same position as vertebrates, but also at C-8, 9, 10 and 12 (Taylor, 1971 and MacDonald *et al*, 1983).

Interestingly, the most abundant pregnane identified in our study, 5â-pregnane-3â and 5-Prgnane 3â was detected in the liver cell culture supernatant by HPLC (See Table 1). Based on analysis of biliary secretion in human and rat, the liver rapidly reduces progesterone to mostly 5â-pregnanes and a lesser amount of 5á-pregnanes (Eriksson *et al,* 1968, 1970). liver of mammals contains both 5á- and 5â-reductases, but it is not clear what determines the reduction of progesterone to one isomer over the other. The most abundant faecal pregnane found in human pregnancy, 5â- pregnane-3α, 20α-diol (Eriksson *et al,* 1970) was also the most abundant pregnane identified in the feces of a non-pregnant Sumatran rhinoceros (Heistermann *et al*, 1998) and in the Indian rhinoceros(Schwarzenberger *et al*, 2000). Lance *et al* (2001) reported 5á-pregnane-3â, 20á-diol, detected in the RIA from faeces. Schwarzenberger *et al* (1993) reported 5á-pregnane-3,20-dione, 5á-pregnane-3â-ol-20-one and 5α-pregnane-3â-ol-20-one as

the most abundant progesterone metabolites in the faeces of black rhinoceros. In the liver cells of water buffalo, 41.91% of the pregnanes by mass were in the 5â-configuration and 3.56% were in the 5á-configuation.

In conclusion, therefore, it seems probable that the conversion rate of progesterone metabolite in liver cells of buffalo decreased with incubation of 5 to 10µg/ml of progesterone concentration during 12 hrs of incubation. As the liver is probably the major site of progesterone metabolism and compounds given orally pass through the liver before entering the general circulation, the results obtained may well be a true reflection of the fate of these progesterone analogues in the intact animal.

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