Endocrinology-Reproductive Physiology Program

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ABSTRACTS FOR INVITED SPEAKERS

Decidualization In The Primate: Cellular And Molecular Changes

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Implantation in the baboon is similar to that described for the rhesus macaque. Specialized villi; known as anchoring villi, facilitate the attachment of the placenta to the uterine wall and provide the source of the migratory cell population that invade into the maternal endometrium. Coincident with this invasive process, the stromal fibroblasts are enlarged compared to the non-pregnant precursors. Decidualization, which involves the transformation of stromal fibroblasts to decidual cells, is the major change that occurs in the primate endometrium after conception. In primates and rodents, the uterine endometrial stromal cells differentiate to decidual cells following the establishment of pregnancy. Decidual cells play an important role in implantation and provide nutritional support for embryo. Decidual cells are also believed to produce factors that control trophoblast invasion and protect the embryo from maternal immune rejection. During the process of decidualization in the primate, fibroblast-like stromal cells change morphologically into polygonal cells and begin to express specific decidual proteins. This is manifested by the downregulation of α-smooth muscle actin expression and the induction of insulin-like growth factor binding protein-1 (IGFBP-1). Previous studies in the baboon have clearly demonstrated that IGFBP-1 gene expression in the endometrium is a conceptus mediated response. Subsequent studies *in vitro* established that IGFBP-1 gene expression in decidualizing stromal fibroblasts requires the presence of both hormones and cAMP. This induction is associated with a concomitant decrease of α-smooth muscle actin expression *in vivo* and *in vitro*. Since IL-1β is expressed both in the progestational endometrium and in trophoblast cells we evaluated IL-1β as one possible factor that could influence differentiation of stromal cells into decidual cells. IL-1β has been reported to be actively involved in fetal-maternal interactions, but its role in decidualization has not been clarified. In addition, IL-1 can modulate changes in the cytoskeleton and induce cyclooxygenase-2 (COX-2) gene expression. Our data would suggest that IL-1β activates a signaling pathway that induces COX-2 expression. COX-2 in turn increases PGE_2 which can increase intracellular cAMP via activation of the EP2 and EP4 receptor. The cAMP in synergism with progesterone results in the induction of IGFBP-1 expression. Induction of IGFBP-1 in these cells is transcriptionally regulated by FKHR and HOXA10 which together activate the IRE on the IGFBP-1 promoter. Coincident with the induction of COX-2, IL-1β also induces metalloproteinase-3 (MMP-3) expression in stromal fibroblasts. We hypothesize that the local action of MMP-3 dissociates the surrounding extracellular matrix resulting in the loss of focal adhesion complexes and the alteration in the actin cytoskeleton. This dissociation is the necessary pre-requisite for decidualization and IGFBP-1 induction. In summary, our studies have demonstrated that a coordinated sequence of events that require a signal from the conceptus are necessary to induce stromal cell differentiation and decidualization. We suggest that these changes are critical to ensure prolonged maintenance of endometrial function during gestation and facilitate trophoblast invasion.

What's Wrong With Reproductive Cloning?

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Everyone agrees that it is premature to attempt cloning of a fully formed human being at this point, at the least because of uncertainty about biologic risk to the offspring. The contentious issue is whether there are any compelling moral arguments against the procedure if it were shown to have acceptable safety. The moral arguments that have been commonly raised will be reviewed, with analysis suggesting that none of them offers a compelling reason to prohibit the procedure.

CD133 - a Pluripotent Stem Cell Marker

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Recent progress in stem cell research indicated that adult stem cells maintain a high degree of plasticity for multilineage cell differentiation.
In blood, CD133 is known as a marker for primitive hematopoietic stem and progenitor cells. Recent studies showed that CD133 has a wider potential in the area of stem cell plasticity that is not restricted to hematopoiesis. This presentation will give an overview regarding research conducted with CD133 in the area of non-hematopoietic cell differentiation such as mesodermal and ectodermal development as well as potential future applications in the field of adult stem cell research.

ABSTRACTS FOR ORAL PRESENTATIONS

2,3,7,8-Tetrachlorodibenzo-*P*-Dioxin Inhibits Prostatic Epithelial Bud Formation In C57BL/6J Mouse Fetus Without Interrupting Androgen Signaling Pathway In Urogenital Sinus

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One of the most sensitive responses to *in utero* and lactational TCDD exposure is a reduction of ventral, dorsolateral, and anterior prostate weight, which occurs with a maternal dose (5 μg/kg of body weight) on gestation day (GD) 13 in C57BL/6J mice. We found that decrease in prostate weight in adulthood correlated with inhibition of urogenital sinus (UGS) prostatic epithelial bud formation by TCDD exposure during gestation, and that TCDD acted directly on the UGS to inhibit androgen-stimulated epithelial bud formation. Prostate development is elicited by testicular androgen, which acts exclusively on androgen receptor (AR) in mesenchymal cells of the UGS to induce outgrowth of epithelial prostatic bud into surrounding mesenchyme. Any interruption of androgen signaling pathway results in impairment of prostate development. In this study, we investigated whether TCDD interrupts androgen signaling pathway in the UGS to cause inhibition of prostatic epithelial bud formation using an *in vitro* culture system. Prostatic epithelial budding occurred in GD 14 male UGS cultured *in vitro* for 5 days in the presence of 10^{-8} M dihydrotestosterone (DHT). In contrast, inhibition of prostatic bud formation was found in UGS treated with either 10^{-5} M hydroxy-flutamide (OH-flutamide, a well-known AR antagonist) or 10^{-9} M TCDD in the presence of 10^{-8} M DHT. To determine whether TCDD inhibits the androgen signaling pathway, primary mesenchymal cells were prepared from GD 14 male UGSs cultured in the presence of 10^{-8} M DHT with 0.1% DMSO or 10^{-9} M TCDD for 3 days, transiently transfected with androgen-responsive luciferase reporter (MMTV-luciferase reporter plasmid), and treated with 0.1% EtOH (vehicle), 10^{-8} M DHT, 10^{-8} M DHT + 10^{-5} M OH-flutamide, or 10^{-8} M DHT + 10^{-9} M TCDD. In both DMSO-exposed and TCDD-exposed groups, we found that 1) luciferase activity in mesenchymal cells treated with 0.1% EtOH (vehicle for DHT) was at the background level, 2) DHT significantly increased luciferase activity about five-fold in comparison to vehicle group, 3) OH-flutamide reduced luciferase activity to the background level even in the presence of DHT, and 4) TCDD did not inhibit androgen-dependent luciferase activity in mesenchymal cells. Androgen-dependent gene expression was also analyzed by quantitating mRNA level for AR and 5α-reductase-type II using real-time PCR. GD 14 UGSs cultured *in vitro* treated with vehicle or 10^{-9} M TCDD in the presence of 10^{-8} M DHT for 3 days were used to evaluate the abundance of AR and 5α-reductase-type II mRNA. It was found that expressions of the androgen dependent genes were not affected by TCDD exposure. These results suggest that TCDD does not have anti-androgenic effects on the UGS. In conclusion, TCDD inhibits UGS prostatic epithelial bud formation, not by interruption of androgen signaling pathway. (Supported by NIH Grant ES 01332)

Ovine Uterine Blood Flow Responses To The Estrogen Receptor Antagonist ICI 182,780

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Estrogen increases uterine blood flow (UBF). Both the follicular phase and pregnancy are physiological states with high levels of circulating estrogen. During these times, estrogens bind intracellular receptor proteins, which up-regulate estrogen receptor (ER). The majority of UBF studies to date have been performed on ovarectomized (ovx) animals using pharmacological doses of exogenous estrogen. We have chosen to study estradiol-17β ($E_2\beta$) and its response to an estrogen antagonist in order to examine UBF in ovx, late pregnant sheep and during synchronized ovarian cycles in intact, cycling sheep. ICI 182,780 (ICI) is thought to be a pure steroidal E2β antagonist, or antiestrogen which blocks estrogen action by competing with estrogen for receptors present in the nuclei of estrogen responsive tissue. **Purpose:** To determine if endogenous and exogenous estrogen increase UBF in a similar fashion via activation of an estrogen receptor. Using a previously developed intact cycling animal model, we are studying the effects of the estrogen receptor antagonist ICI and its effects on UBF during synchronized ovarian cycles in sheep, as well as in ovx and late pregnant sheep. **Methods:** Sheep were surgically instrumented with bilateral uterine artery blood flow transducers, uterine and femoral artery catheters. Ovx animals were infused into one uterine artery with vehicle (EtOH) or ICI for ten minutes before estrogen was given (1 μg/kg *bolus*) into the vena cava. Fifty minutes after the $E_2\beta$ injection the vehicle or ICI infusion was stopped and UBF was recorded for an additional hour. Infusion rates for ICI were between 0.1 and 3.0 μg/min at 0.103ml/min (n=8). Late pregnant ewes were given similar doses of ICI with a 60min unilateral infusion (n=6). Intact, cycling sheep were implanted with a vaginal progesterone controlled internal drug release (CIDR; 0.9g) device for 7 days. On Day -1, animals received 7.5mg of $PGF_{2\alpha}$ I.M., 2x- 4 hours apart. On Day 0, the CIDR was removed and 1000 IU PMSG was given I.M. ICI doses of 1ug/min and 2ug/min at an infusion rate of 0.097ml/min were given unilaterally at approximately 50hrs, or when UBF reached peak levels (n=3). **Preliminary Results:** ICI decreased the ipsilateral UBF response to endogenous and exogenous $E_2\beta$ in ovx, pregnant and intact cycling ewes in a time and dose dependent manner. The ipsilateral effect was greater in all groups, however, the higher the concentration of ICI given the higher incidence of a contralateral reduction in UBF occurred. In addition, ICI did not significantly alter either mean arterial pressure or heart rate in the absence or presence of $E_2\beta$. **Conclusion**: The endogenous $E_2\beta$ induced increases in UBF during late pregnancy and the follicular phase and the exogenous $E_2\beta$ induced increases in UBF in the ovx animal are decreased in a dose related fashion by the specific estrogen receptor antagonist ICI 182,780 via an estrogen receptor-mediated mechanism.

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Effects Of Varying Intervals From Dominant Follicle Emergence To Progestin Removal On Follicular Dynamics And Estrus Synchronization In Beef Cattle.

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The objective of the experiment was to determine if varying the interval from emergence of a new follicle to the end of an estrus synchronization treatment affected the synchrony of estrus. On Day 6 to 8 of the estrous cycle non-lactating beef cows were fitted with a progesterone releasing intravaginal device (CIDR; n=49). At CIDR insertion each cow received an i.m. injection containing either 1 mg estradiol-17β and 100 mg progesterone (EP) or 100μg of GnRH. CIDRs remained in place for 7 or 9 d. In addition, one half of the animals in each subgroup were treated with 37.5 mg PG at CIDR insertion to regress the corpus luteum (CL). All cows received 25 mg of PG 24 h prior to CIDR removal. HeatWatch was used to monitor estrus activity. Ovarian follicular development was monitored by ultrasonography. Data were analyzed as a 2x2x2 factorial with: EP or GnRH; 7- or 9-d CIDR; and CL regressed or present as main effects. The interval from follicle emergence to CIDR removal was greater following GnRH treatment or in animals fitted with a CIDR for 9 d. The longer interval from follicle emergence to CIDR removal increased dominant follicle (DF) size at CIDR removal. Cows with a larger DF at CIDR removal tended to exhibit estrus earlier, but no differences in the synchrony of estrus were detected. Cows with the CL regressed at CIDR insertion had a larger DF at CIDR removal and exhibited estrus earlier, however, synchrony of estrus was not affected. Treatments altered the interval from follicle emergence to progestin removal and affected follicular dynamics, but did not improve the synchrony of estrus.

	E +P	GnRH	CIDR 7	CIDR 9	Saline	PG	Pooled SE
Emergence to CIDR removal (d)	4.7[a]	6.6[b]	4.8[a]	6.5[b]	5.5	5.8	0.2
DF size at CIDR removal (mm)	11.3[a]	13.4[b]	11.8[a]	13.0[b]	11.5[a]	13.2[b]	0.4
CIDR removal to Estrus (h)	55.3	49.8	56.4	48.8	58.3[a]	46.8[b]	3.4

*Least-square means within each row and main effect with uncommon superscripts differ (P<0.07).

Lack Of Direct Evidence For ERK 1/2 Involvement In ATP Stimulated Prostacyclin Production In An Ovine Uterine Artery Endothelial Cell Culture Model (UAEC): Implication For A PLC And IP3R Mediated Calcium Dependent PGI2 Production.

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To afford the 20% redirection of blood flow to the utero-placental unit during pregnancy, many vascular alterations need to occur. Shear stress, hypoxia and administration of certain agents such as growth factors and steroid hormones have been associated with angiogensis of certain vascular beds and the local production of endothelial derived vasoactive agents such as Prostacyclin (PGI2) to accomodate the increased utero-placental blood flow. Prostacyclin is formed after liberation of Arachidonic Acid (AA) from the plasma membrane by cPLA2, and the subsequent actions of Cyclooxygenase I and PGIS. The rate limiting step in PGI2 production is usually the first hormone sensitive step, the liberation of AA from the plasma membrane through the actions of cPLA2. The activity of cPLA2 has been shown to be regulated by an increase in Ca^{2+} and/or phosphorylation at S505. In turn S505 has been shown to be phosphorylated by a prototypical member of the MAPK family, ERK1/2. ATP is unique in our culture system because it is one of a few observed agonists to stimulate a detectable increase in intracellular Ca^{2+} as well as signal through ERK1/2. We have employed our UAEC model that retains certain observed pregnancy specific functional changes, including a pregnancy enhanced eNOS activation and enhanced coupling of agonist to ERK1/2 activation, to investigate the effects of ATP treatment on PGI2 production and the activation of intracellular signaling cascades implicated in the regulation of key enzymes involved in PGI2 production. We have preliminary data showing ATP stimulates an increase in PGI2, and that the increase in PGI2 shows a trend towards dose dependent inhibition with the PLC inhibitor, U73122 (.01-20uM) as well as the IP3 receptor antagonist 2-APB (1-50uM). The ATP stimulation of ERK1/2 was abolished only at high concentrations (100uM) of 2-APB, and (20uM) U73122. There was no observed trend toward dose dependent inhibition with either antagonist in investigating ERK1/2 activation. ATP does not increase the level of phosphorylation at S505 and pretreatment with either antagonist has no discernable effect on the phosphorylation state of this residue by Western Analysis. It appears, preliminarily, that the ATP stimulation of PGI2 involves some PLC activity and likely a subsequent IP3 receptor mediated release of Ca^{2+}. The dependence of PGI2 production on ERK1/2 activation in response to ATP is in question after the observation that ATP does not cause an increase in S505 phosphorylation and that the ATP activation of ERK1/2 is only inhibited at high concentrations of both 2-APB and U73122. Therefore, we are lead to believe the ATP stimulation of PGI2 is dependent on a rise in intracellular Ca^{2+}, and ERK1/2 activation is only coincidentally involved, if at all. *Supported by USDA 0002159, NIH-HL56702, HL49210.*

Threonine 495 Phosphorylation Is Not Necessarily Associated With Inhibition Of eNOS Activity.

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We have isolated and cloned a full-length cDNA encoding ovine endothelial nitric oxide synthase. In vivo, eNOS is constitutively expressed and is negatively regulated by caveolin and positively regulated by Ca^{2+}/calmodulin and the chaperone Hsp90. In addition, regulation of eNOS activity may be sensitive to intracellular Ca^{2+} concentration and multiple phosphorylation events. Thr 495 and Ser 1177 (human) phosphorylations have been investigated most intensely. Thr 495 phosphorylation associates with decreased eNOS enzymatic activity presumably through reduced interaction with Ca^{2+}/calmodulin. In contrast, Ser 1177 phosphorylation associates with increased eNOS activity. This study investigates eNOS activity during treatment with Ca^{2+} ionophore or phorbol ester. The relative phosphorylation-state of two eNOS amino acid residues was also determined. COS-7 cells were transfected with ovine eNOS cDNA (pBK-CMV) using GeneJammer (Stratagene). Following transient expression of eNOS, cells were treated with either a Ca^{2+} ionophore, A23187 (10 mM), or phorbol ester, phorbol-12-myristate-13-acetate (PMA, 10 nM) or both. eNOS activity was assayed by ^{3}H-arginine to ^{3}H-citrulline conversion in intact cells. Phosphorylation-state specific antibodies were used for immunoblotting of eNOS, Akt and Erk 1/2, followed by non-phospho specific antibodies. A23187 increased eNOS activity (805±120 pmol/well over vehicle control, $P<0.01$) and Ser 1177 phosphorylation while decreasing Thr 495 phosphorylation. TPA treatment did not change eNOS activity and increased both Ser 1177 and Thr 495 phosphorylation. Combined A23187 and TPA induced eNOS activity (660±137 pmol over vehicle control/well, $P<0.01$) and also increased both eNOS Thr 495 and Ser 1177 phosphorylation. All treatments increased phosphorylation of Akt and Erk 1/2. In summary, Ca^{2+}-stimulated eNOS activity is not attenuated by phorbol ester treatment, despite significant phosphorylation of Thr 495, previously shown to block eNOS and Ca^{2+}/calmodulin interaction. Phosphorylation or other post-translational modifications of eNOS may occur that allow eNOS to remain active in the face of reduced Ca^{2+}/calmodulin binding. The fact that Ca^{2+} ionophore treatment activates Akt, Erk 1/2, and possibly other kinases argues that additional modifications of eNOS may be taking place and must be studied further. Supported by USDA-0002159, NIH-HL64601.

Hormonal And Follicular Responses Associated With A Natural And GnRH-Induced Ovulation In Heifers

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Hormonal changes near the time of ovulation include the preovulatory LH and FSH surges, a rapid decline in estradiol, and a second, periovulatory surge of FSH. Follicle changes include ovulation of the dominant follicle and emergence of a new follicular wave. Our objective was to compare changes in plasma FSH, LH, estradiol, and total α-inhibin, in addition to antral follicle development, during a natural and GnRH-induced ovulation. Heifers were treated with a single injection of saline (n=7) or 100 µg of GnRH (gonadorelin; n=6) 36 h after prostaglandin ($PGF_{s\alpha}$). Ultrasound scans and hourly blood samples were acquired 6 h before treatment until 12 h after detection of an 8.5 mm follicle in the new follicular wave. GnRH treatment reduced the time to ovulation. In GnRH-treated heifers, the preovulatory LH and FSH surges were 3 h shorter and peak concentrations of LH and FSH were 51% and 58% greater, respectively, compared to control. Normalized to the time when LH concentrations surpassed 4.5 ng/ml, estradiol concentrations declined similarly in each group and nadir concentrations were evident within 12 h. Normalized to this same time point, the periovulatory FSH surge commenced earlier in GnRH (9.5 ±0.9h) compared to control (14.0 ±0.7h). Regardless of treatment, substantial variation in the pattern and duration of the periovulatory FSH surge existed among individual heifers. All endpoints pertaining to the periovulatory FSH surge were unaffected by treatment. In reference to onset of the periovulatory FSH surge, the time to follicular wave emergence, detection of an 8.5 mm follicle, and the number of follicles growing larger than 4, 5, or 8.5 mm, were similar between treatments. The results indicate that inducing ovulation with a single GnRH injection alters the pattern of the preovulatory LH and FSH surges; however, the decline in estradiol, the periovulatory surge of FSH, and growth of follicles in the postovulatory follicular wave remain unchanged.

ABSTRACTS FOR POSTER SESSION

Effect Of Different Cell Types And Cell Lines On Development Of Bovine Nuclear Transfer Embryos

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The objective of this study was to examine the effect of donor cell type and cell line (chondrocytes, cumulus cells, fetal and adult fibroblasts) on development of bovine nuclear transfer embryos. Cumulus cells were isolated from in vitro matured oocytes, adult chondrocytes and fibroblasts were isolated from slaughtered animals and fetal fibroblasts were obtained from 45-60 day fetuses from a local slaughterhouse. All cell types were cultured for 1-16 passages before nuclear transplantation. Nuclear transfer experiments were conducted as described by Dominko et al. (Biol Reprod 1999; 60 (6): 1496-502). Recipient oocytes were matured in vitro, stripped of cumulus cells and enucleated at 18-20 hours post maturation (hpm). Enucleation was performed under ultraviolet light and oocytes were stained with Hoechst 33342 (5μM) to confirm complete removal of chromatin. A total of 17 different cell lines from four different cell types (3 bovine adult fibroblast, 2 chondrocyte, 9 cumulus cell and 3 fetal fibroblast lines) were employed in our study. Overall, 3123 NT embryos were produced in 117 trials using one cell line in each trial. Data were analyzed by ANOVA and means were compared by protected LSD. Results are summarized in Table 1.

Cell Line	n	Fusion % (N)	Cleavage % (N)	Blastocyst % (N)	ET	P	LB
BAF1	72	83.6±7.6 (03) ^{c,d}	63.7±9.4 (03) ^{a,b}	24.6±6.6 (03) ^{a,b,c}	9	1	
BAF2	84	94.8±6.6 (04) ^{d}	65.5±8.2 (04) ^{a,b}	12.2±5.8 (04) ^{a}	0	0	
BAF3	86	49.7±7.6 (03) ^{a}	74.3±8.2 (04)^{b}	19.6±5.8 (04) ^{a,b}	0	0	
BCh5	67	84.8±9.4 (02) ^{c,d}	75.5±11.5 (02)^{b}	19.3±8.1 (02) ^{a,b}	0	0	
BCh6	914	86.2±2.3 (34) ^{c,d}	61.2±2.8 (34) ^{a,b}	20.1±2.0 (34) ^{a,b}	20	1	
BCm7	109	96.0±6.6 (04) ^{d}	60.9±8.2 (04) ^{a,b}	37.9±5.8 (04) ^{c}	14	1	
BCm8	727	88.8±2.5 (28) ^{c,d}	72.6±3.1 (28)^{b}	26.5±2.2 (28)^{b,c}	54	5	
BCm9	80	91.5±7.6 (03) ^{c,d}	57.0±9.4 (03) ^{a,b}	27.5±6.6 (03)^{b,c}	2	0	
BCm10	165	76.3±5.9 (05) ^{b,c}	59.7±7.3 (05) ^{a,b}	19.6±5.1 (05) ^{a,b}	0	0	
BCm11	134	88.9±5.9 (05) ^{c,d}	60.5±7.3 (05) ^{a,b}	19.0±5.1 (05) ^{a,b}	2	0	
BCm12	89	88.2±7.6 (03) ^{c,d}	58.0±9.4 (03) ^{a,b}	19.4±6.6 (03) ^{a,b}	4	0	
BCm13	79	88.0±7.6 (03) ^{c,d}	63.1±9.4 (03) ^{a,b}	20.6±6.6 (03) ^{a,b}	2	0	
BCm14	117	84.5±6.6 (04) ^{c,d}	63.8±8.2 (04) ^{a,b}	28.4±5.8 (04)^{b,c}	2	0	
BCm15	90	81.9±6.6 (04) ^{b,c,d}	64.4±8.2 (04) ^{a,b}	17.3±5.8 (04) ^{a,b}	5	0	
BFF16	25	100.0±9.4 (02)^{d}	40.1±11.5 (02) ^{a}	20.0±8.1(02) ^{a,b}	2	0	
BFF17	57	60.3±9.4 (02) ^{a,b}	69.0±11.5 (02)^{b}	42.1±8.1 (02) ^{c}	0	0	
BFF18	228	83.0±4.7 (08) ^{c,d}	67.8±5.8 (08)^{b}	16.4±4.1 (08) ^{a}	7	3	3

Table 1: The effect of individual cell lines on pre- and post-implantation development of bovine nuclear transfer embryos. Different superscripts within the same column indicate statistical differences ($p<0.05$) BAF: bovine adult fibroblasts, BCh: bovine chondrocytes, BCm: bovine cumulus cells, BFF: bovine fetal fibroblasts. ET: embryo transfer, P: pregnancy, LB: Live birth.

Our results indicate that there are significant differences among individual cell lines, but not cell types in terms of pre- and post-implantation development of NT embryos. Our results also suggest that higher developmental rate of cumulus cells and chondrocytes to the blastocyst stage were not correlated with the frequency of pregnancy establishment and development to term. Only one bovine fetal fibroblast line (BFF18) has resulted in 43% pregnancy establishment and all three pregnancies were resulted in live birth.

Comparative Expression of Estrogen Receptors and Endothelial Nitric Oxide Synthase in the Ovine Artery Endothelium.

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Endothelium as well as the outer layer of vascular smooth muscle (VSM) has been shown to contain estrogen receptors and bind estrogen with high affinity. This is significant because estrogen is believed to have a multifunctional role as a cardiovascular protectant. Estrogen elicits a rapid vasodilatory response followed by long term prevention of artherosclerosis by inhibition of the response to vascular injury. The direct action of estrogen on endothelial cells as well as VSM mediates this effect. Aside from its role as a cardiovascular protector, estrogen also plays a critical role in regulating uterine blood flow during pregnancy. The 20-50 fold increase in uteroplacental blood flow seen during normal pregnancy in sheep occurs, in part, at the level of the uterine artery endothelium where estrogen stimulates the expression of endothelial nitric oxide synthase (eNOS) resulting in increased production of the potent vasodilator nitric oxide (NO).

To determine if the specific estrogen-induced increase in eNOS is unique to the uterine vascular bed, and limited only to the endocrine conditions of pregnancy, we have conducted a study of vessels from a variety of systemic tissues during different stages of the reproductive cycle. Arteries were collected from uterine, omental, mammary, renal, coronary, and placental vascular beds (n=5-9). For the purposes of endocrine status comparison, each of these vessels was collected from luteal and follicular phase sheep (except placental), as well as pregnant sheep. Immediately following surgical removal of the vessels, the endothelial layer was mechanically removed and snap frozen in lysis buffer for later analysis. Using Western blotting, protein lysates were probed with antibodies for estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), eNOS, PECAM-1 (an endothelial cell marker), and smooth muscle myosin (SMM, an indicator of muscle cell contamination).

eNOS was found to be significantly elevated in the uterine artery endothelium during the follicular phase (4.2 fold) as well as pregnancy (5.8 fold) when compared to luteal samples. No other vessel studied showed an increase in eNOS. ERα did not change in any tissue studied during these endocrine conditions. ERβ showed a very modest increase (1.6 fold) during pregnancy in the uterine, mammary, and coronary endothelium. However, considering eNOS increases more than 4 fold during the follicular phase, when ERβ is unchanged, it is not likely that an increase in ERβ is required. This suggests that the regulation of eNOS by ERs is through a non-genomic signaling pathway that does not require increased translation of receptor protein. PECAM-1 was readily detectable in all samples confirming the presence of endothelium. SMM protein levels were consistent in all tissues validating the relative purity of the endothelial samples. In conclusion, endocrine related changes in eNOS are localized specifically the uterine artery endothelium.

Differentiated Expression of VEGF, EG-VEGF, and VEGF Receptors in Human Placentas from Normal and Pre-eclamptic Pregnancies.

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In normal pregnancy angiogenesis and vasodilation are critical for the increased placental blood flows which is directly correlated with fetal growth and survival as well as neonatal birth weights and survivability. Abnormal vascular growth and impaired vasodilation, however, may result in abnormal pregnancy conditions such as pre-eclampsia (PE), a hypertensive disorder in 10-13% of first pregnancies and in 5-7% of subsequent pregnancies. The pathogenesis of PE is thought to act at three levels: defective placentation, placental ischemia, and endothelial cell dysfunction. To date, although dysfunction of vascular endothelial cells is considered to be a key factor which results in these abnormal pregnancy conditions, the basic biochemical and molecular mechanisms that lead to endothelial dysfunction in the abnormal pregnancies remain unclear. In this study, we examined differentiated expression of VEGF, endocrine-gland-derived VEGF (EG-VEGF), and VEGF receptors (VEGF receptor-1 [VEGFR-1] and -2 [VEGFR-2], and neuropilin-1 [NP-1] and -2 [NP-2]) in human placentas from normal (N) and PE pregnancies using quantitative real-time PCR. We observed slight increases (54 and 44%) in VEGF and EG-VEGF expression in PE vs N placentas. Expression of VEGFR-1, but not the other receptors (VEGFR-2, NP-1 and NP-2), increased 4 fold in PE placentas when compared with normal placentas. Expression of all four VEGF receptors in placentas was confirmed using immunohistochemistry. The localization of VEGF receptors in placentas was similar among four VEGF receptors: primarily present in syncytiotrophoblasts and endothelial cells of villous capillaries and large vessels. No significant difference in staining intensity was detected between the N and PE placentas examined. Thus, we have demonstrate that VEGFR-1 expression is increased in pre-eclamptic placentas, suggesting VEGFR-1 may play an important role in pre-eclampsia.

Changes In FSH Concentrations In Relation To The Follicular Population During Wave Development In Mares

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Knowledge of the association between circulating FSH concentrations and follicle development is needed for development of optimal superovulation protocols. An FSH surge associated with follicular wave emergence at about mid-cycle in mares attains peak concentrations when the largest follicle reaches approximately 13 mm. Thereafter, concentrations decrease concurrent with the development of multiple follicles of the wave. The extent of involvement of the cohort of follicles in regulating this FSH decline is unknown. The hypotheses was tested that several growing follicles >13 mm of a wave participate in the FSH decline. Cycling pony mares were randomly assigned to groups based on the number of follicles >10 mm retained in their ovaries. In brief, a new follicular wave was induced 10 days after ovulation by ablating all ovarian follicles >5 mm using ultrasound-guided transvaginal follicle aspiration. Thereafter, ablations were done when the largest follicle reached 10 mm to retain none (n=15), the largest (n=12), the 3 largest (n=15), or all (n=10) follicles >10 mm of the ablation-induced wave. Transrectal ultrasonography to monitor follicular development and blood sampling were done daily. Hormonal data were normalized to the day the largest follicle attained or was expected to attain 13 mm (day 0) and were analyzed by split-plot ANOVA from day -4 to day 8. A treatment by day interaction ($p<0.01$) for mean FSH concentrations was due to a progressive decrease in concentrations after day 0 in the groups in which at least 1 follicle was retained, while concentrations in the group with no follicles retained remained elevated. The FSH decline was more precipitous in the groups with the 3 largest or all follicles retained than in the group with only the largest follicle retained, as indicated by lower ($p<0.05$) FSH concentrations on day 2 in the first two groups. However, all 3 groups with follicles retained had reached similar FSH levels by the time their largest follicle was approximately 25 mm. No differences in plasma estradiol levels were detected among groups, although an increase was observed (day effect, $p<0.05$) when the largest follicle in the groups with follicles retained exceeded 20 mm. The mean profiles of LH concentrations were similar among groups with a gradual increase over the 13-day period (day effect, $p<0.05$).

In conclusion, a cohort of at least 3 growing follicles initially participated in the decline in FSH concentrations associated with a follicular wave. Thereafter, at a mean of 23 mm, a single follicle accounted for the depressed FSH concentrations.

The Effects Of Scrotal Insulation On Sperm Morphology And In Vitro Fertilization

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ABSTRACT- The effect of scrotal insulation for 48 hr on bull sperm morphology as assessed by Fourier harmonic analysis and in vitro fertility as assessed by ability of sperm to penetrate oocytes, the ratio of oocytes cleaved at 32/48 hr post insemination, and number of nuclei after 135 hr of culture. Analysis of sperm morphology used a quality control chart approach comparing semen collections obtained before and at 2 to 3 day intervals after scrotal insulation (ASI). The sum of the Fourier harmonic amplitude variances were used as measures of the variation in sperm nuclear shape. The variation in sperm nuclear shape increased (p0.05) for collections (days ASI)) with sperm that were undergoing epididymal transit (day 2) and spermiogenesis (day 14), meiosis (days 42 and 47) at the time of insulation. These changes in nuclear shape variation would be indicative of reduced fertility. When examining the in vitro fertility of the same semen samples, no effect was found on fertilization. Timing of first cleavage was evaluated as faster cleaving embryos are more likely to develop to the morula/blastocyst stage. The ratio of cleavage at 32/48 hr was decreased (p0.05) for collections (days ASI) with sperm undergoing epididymal transit (day 2), spermiogenesis (day 14, 23), meiosis (days 30, 33, 35, 37, 47) and spermatocytogenesis (days 49, 61 and 66) at the time of insulation. The number of nuclei in embryos after 135 hr of culture were reduced for collections (days ASI) from 191 nuclei (day 0) to 132 (day 14) and 162 (day 26). There were effects on sperm in vitro fertility for sperm undergoing all stages of development at the time of scrotal insulation. The Fourier harmonic analysis identified a variety of changes to the variation in sperm shape but it was best able to identify changes in sperm at days 2, 14 and 47 that were related to reduced in vitro fertility. In particular, day 14 sperm undergoing spermiogenesis had increased variation in shape, delayed time to first cleavage, and reduced number of nuclei by 135 hr of culture. Supported by USDA 98-02163.

BMP-4 Induces Trophoblast Differentiation In Human ES-Cell Derived Embryoid Bodies.

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The earliest differentiation event in mammalian embryonic development is the determination of the trophectoderm. Chorionic gonadotropin (CG) secretion by trophoblasts at the time of implantation is an essential signal for pregnancy recognition in all primates. Although insights into early placental development have been provided by mouse genetic models, the relevance to human development remains to be systematically investigated. In order to begin to investigate the signals that cause differentiation of embryonic cells into specific trophoblast linages, we used embryoid bodies (EBs) prepared from human embryonic stem (hES) cells as an *in vitro* model of early human development. hES cells (line H1.1) were grown in suspension culture for 10 days to form EBs. EBs were treated with varying concentrations (100ng/ml, 30ng/ml and 10ng/ml) of bone morphogenetic protein -4 (BMP-4) and supernatant was collected every 48 hours. After 96 hours EBs were washed and resuspended in media without BMP-4. The secretion of hCG, progesterone, and estrogen increased significantly (> 10-fold) when EBs were exposed to all levels of BMP-4. Very low hormone production was noted in untreated EBs. EBs produced substantial amounts of hCG, progesterone, and estrogen throughout exposure to BMP-4, although the hormonal response began to decline during the subsequent 48 h of BMP-4 withdrawal. We have used two different trophoblast cell lines (JEG-3 and BeWo) to determine whether BMP-4 also stimulates endocrine function of differentiated trophoblasts. The studies demonstrated little if any response in hormone secretion with these cell lines.

Summary: Our lab developed a paradigm using EBs to study the role of BMP-4 in early stages of embryonic development. EBs were exposed to different doses of BMP-4 at 48 hours intervals. In some experiments, BMP-4 treated EBs secreted the highest levels of hCG and progesterone, however the response was not consistent in all experiments.

The response pattern to BMP-4 is complex and differs among experiments; however the observation that hCG and Progesterone responses to BMP-4 tend to be similar within an experiment suggests that trophoblast differentiation and/or activity is altered in EBs.

Enteral Insulin-Like Growth Factor-I (IGF-I) Stimulates Erythropoiesis In Suckling Rats.

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Background: IGF-I and IGF-II are potent growth factors involved in perinatal growth and development. Parenteral IGF-I has been shown to stimulate erythropoiesis, whereas IGF-II's role in erythropoiesis is inadequately studied. Although IGF-I and IGF-II are both present in mammalian milks, the role of milk-borne IGF's in erythropoiesis has not been examined. Cell culture studies support 2 potential mechanisms involved in IGF-I's stimulation of erythropoiesis: by stimulating Epo production and by stimulating erythroid maturation at later stages of differentiation. Investigators have also speculated that IGF-I stimulates cellular iron delivery by upregulating the transferrin receptor. We investigated whether artificial feeding of IGF-I and IGF-II to suckling rats stimulates erythropoiesis. Methods: 8-12 day-old rats underwent gastrostomy and were fed utilizing an artificial feeding system with rat milk substitute (RMS), devoid of growth factors. We studied rats fed RMS+IGF-I, RMS+IGF-II, or RMS. Blood and bone marrow were collected to measure hemoglobin (Hb), plasma Epo levels, reticulocytes, myeloid:erythroid ratio, mean cell volume, and zinc protoporphyrin (a measure of incomplete iron incorporation into Hb). Results are reported as mean ± SEM) and analyzed by factorial ANOVA. Results: Rats fed RMS + IGF-I had higher hemoglobin (Hb) levels (9.8 ± .3 g/dL), compared to those fed RMS (9.1 ± .2) or RMS + IGF-II (9.1 ± .2), $p<0.05$. After IGF-I supplementation, red blood cell counts (RBC) ($p<0.05$) and microhematocrits ($p<0.02$) were also higher. Plasma erythropoietin (Epo) levels, reticulocytes, and erythrocyte iron incorporation were similar between groups. Plasma iron levels were higher in the RMS + IGF-I group ($p<0.01$). Conclusion: Enteral IGF-I may directly stimulate erythropoiesis through established signaling pathways, but not by increasing Epo production. Because plasma iron levels were higher with IGF-I supplementation, erythropoiesis might also be stimulated by improved iron delivery.

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IP3 Controls Both The Acute And Sustained Phases Of The Calcium Response In P-UAEC But By Distinct Mechanisms

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We have previously demonstrated that uterine artery endothelial cells derived from pregnant and non-pregnant ewes retain differences in vasodilator production through passage 4. Cells derived from pregnant ewes (P-UAEC) produce more nitric oxide (NO) than cells derived from non-pregnant animals (NP-UAEC) when stimulated by AII or ATP. One way eNOS (the enzyme that catalyzes the conversion of L-arginine to L-citruline and NO) can be activated is by a rise in the intracellular free Ca^{2+}concentration ($[Ca^{2+}]_i$) and it has also been shown that P-UAEC and NP-UAEC show differences in Ca^{2+} signaling. Specifically, P-UAECs respond to ATP with a initial transient peak in $[Ca^{2+}]_i$ followed by a prolonged sustained phase; NP-UAECs do not exhibit a sustained phase. Previous studies have shown that the ATP-induced transient peak in $[Ca^{2+}]_i$ is produced by the release of Ca^{2+} from intracellular stores and is controlled by IP3. Therefore, the aim of this study was to determine the mechanism that controls the sustained phase of the Ca^{2+} response to ATP in P-UAEC. The previous studies had all been performed over a short time interval, thus it was important to further characterize the sustained phase by extending the recording time to 30 minutes. Once a typical response was established, manipulations could be carried out to determine the mechanism by which it is controlled. Research on other cell types which show a biphasic calcium response have revealed that the sustained phase can be due to an influx of extracellular Ca^{2+} after the intracellular stores have been depleted (capacitative entry). In order to determine if this occurred in P-UAEC, the cells were stimulated with ATP in the absence of extracellular Ca^{2+}. The sustained phase was completely abolished in Ca^{2+}-free media but the initial peak was unaffected. Further experiments revealed that the sustained response could be rescued if extracellular Ca^{2+} were added back to the media. In addition, if 2APB (an IP3R antagonist) were added to the media before the extracellular Ca^{2+} was reintroduced, it could block the rise in $[Ca^{2+}]_i$. From this data it can be inferred that extracellular Ca^{2+} does not enter the cytoplasm directly but rather it is channeled into the intracellular stores and is then released into the cytoplasm.

Zinc Protoporphyrn/Heme Ratios In Infants Born To Diabetic Mothers (IDM)

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Introduction: Infants born to mothers with diabetes mellitus (IDM) are at risk for tissue iron deficiency in the perinatal period. Tighter glycemic control during pregnancy has been shown to improve neonatal outcomes. It is not known if improved glucose control is associated with improved iron status. Whole blood zinc protoporphyrin/heme (ZnPP/H) measures incomplete iron incorporation into hemoglobin as zinc substitutes for iron in the protoporphyrin ring. In mature patients, ZnPP/H measures iron deficient erythropoiesis, a state that occurs prior to the development of tissue iron deficiency. We hypothesized that ZnPP/H ratios would be higher in IDM and would correlate to glycemic control. Methods: At birth, we measured ZnPP/H ratios in 110 healthy term infants and in 32 IDM. We measured complete cell counts in IDM and glycosylated Hb levels in their mothers. Patient groups were compared by the Mann-Whitney U test and laboratory parameters were compared by simple regression. Results: ZnPP/H ratios were higher in IDM (127.3±12.6 μM/M) than in normal infants (75.4±2.9 μM/M), $p<0.0001$. ZnPP/H ratios in IDM correlated with red cell distribution width ($p<0.0001$, $R2=.448$), a parameter that rises early in iron deficiency. ZnPP/H ratios did not correlate to red cell count. In 14 instances when maternal glycosylated hemoglobin levels were available, ZnPP/H ratios in IDM did not correlate with glycosylated hemoglobin levels, $p=0.16$, $R2=.157$. Conclusion: ZnPP/H ratios were higher in IDM, compared to normal infants. ZnPP/H ratios in IDM were associated with a measure of early iron deficient erythropoiesis (red cell distribution width), but not associated with the surrogate measure of glycemic control (glycosylated hemoglobin).

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Mechanisms Of Shear Stress Induced Nitric Oxide Synthase (eNOS) Phosphorylation In Ovine Fetoplacental Artery Endothelial Cells.

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Placental blood flow, nitric oxide (NO) level and eNOS expression increase during human and ovine pregnancy. Shear stress, the frictional force exerted on endothelial cells by blood flow, stimulated NO production and eNOS expression in ovine fetoplacental artery endothelial (OFPAE) cells. Our hypothesis is that MEK1/2 and/or PI-3K pathways are involved in shear stress induced eNOS phosphorylation in OFPAE cells. Methods: OFPAE cells were grown at 3 dynes/cm2 in CELLMAX artificial capillary modules. When the cells reach confluence, they were exposed to shear stress of 15 dynes/cm2 for 0, 5, 10, 20 or 30 min. Then the capillaries were perfused with lysis buffer, and Western analysis was performed on the cell lysates for phosphorylated proteins such as eNOS, p38, ERK1/2, and Akt. Based on the time course data, MEK inhibitor UO126 (10 uM) or PI-3K inhibitor LY294002 (50 uM) were introduced into the module 1 hour before shear stress was elevated to 15 dynes/cm2, and the cells were lysed at 0 and 20 min stimulation. Results: Phosphorylation of eNOS on Ser-1177, ERK1/2 and Akt, but not p38 MAP kinase, were further elevated by 15 dynes/cm2 shear stress. UO126 completely blocked the phosphorylation of ERK1/2 at 0 and 20 min at 15 dynes/cm2, but it had no effects on the induction of eNOS phosphorylation on Ser-1177. LY294002 intensively blocked the phosphorylation of Akt at both basal and stimulatory shear stresses, it also inhibited the induction of eNOS phosphorylation on Ser-1177. Conclusion: Shear stress induced rapid eNOS phosphorylation on Ser-1177 in OFPAE cells was mediated by PI-3K pathways.

Shear Stress Regulation of Estradiol-17b (E2b) - Induced Rises in Endothelial Nitric Oxide Synthase (eNOS) Expression in Ovine Uterine Artery Endothelial Cells (UAEC) is Not Associated With Elevations in Estrogen Receptors

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During pregnancy and with estrogen treatment, uterine blood flow (UBF), Nitric Oxide, and eNOS levels are elevated. Increases in UBF are expected to elevate laminar/pulsatile shear stress and thus endothelial eNOS expression. We recently reported that when compared to static cultures, in the presence of basal shear stress, E2b dramatically augments (3-4 fold) the rise in eNOS protein expression in UAEC. Hypothesis: Shear stress will augment estrogen-induced increases of eNOS expression in UAEC by elevating ERa and ERb expression. Methods: UAEC from pregnant sheep were inoculated (4.0x106) into CELLMAX artificial capillary modules and grown at a shear stress of 3 dynes/cm2 until confluent (10-14 days). After 30 min pretreatment with Vehicle vs. E2b (10nM) UAEC were exposed to shear stresses of either 3 (basal) or 15 dynes/cm2 (physiologic range » 12-15 dynes/cm2) for 24 hours. UAEC proteins were eluted and subjected to Western analysis for ERa and ERb expression. Results: Confluence was established by the stabilization of lactate production and the UAECs were seen to grow in a monolayer under light and Scanning Electron Microscopy.

In static culture, E2b did not substantially alter the eNOS expression in UAEC. In contrast, at 3 dynes/cm2, eNOS expression was elevated (3.6 fold of control) in the presence of E2b. Shear stress stimulation of 15 dynes/cm2 alone increased eNOS levels to 4.0 fold of control; this response did not appear to be additive/synergistic in the presence of E2b (5.6 fold of control). Conclusion: Physiologic shear stresses in the absence or presence of E2b increase the expression of eNOS in UAEC. Support by: NIH grants HL49210, HD33255, HL57653, HD38843, HL64601.

In Vitro Systems For Cloning Sheep

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Most reports of producing sheep by nuclear transfer described the use of in vivo matured oocytes. We have developed a system of cloning sheep by nuclear transfer using in vitro matured oocytes. Oocytes were aspirated from slaughterhouse ovaries and COC's were matured in maturation medium (Ptak G et al., Theriogenology 1999;50:1105-1114) for 16 hours. Adult ewe donor cells were recovered from an ear biopsy and cultured in DMEM/F12 (Gibco, Rockville, MD) plus 15% fetal bovine serum (Hyclone, Logan, UT) until confluent, then used in nuclear transfer as described previously (Wells DN et al., Biol Reprod 1997;57:385-393) with modifications. Enucleation of oocytes was performed in calcium free TL HEPES by 17 hours post on-set of maturation (hpm). Donor cell transfer was performed in TL HEPES (Biowhittaker, Walkersville, MD) and the donor cell and oocyte were fused to form cybrids by 19 hpm in 0.25 M sorbitol (SOR) fusion medium (Betthauser et al., Nat Biotech 2000;18:1055-1059). Cybrids were activated at 24 hpm by exposure to 10 µM ionomycin for 4 minutes followed by 4 hours in 2 mM DMAP. NT embryos were cultured in CR2 medium (Rosenkranz CFJ and First NL, Theriogenology 1991;35:266 abst). Up to three blastocysts at day 7 in culture were transferred surgically into each recipient. Day 7 blastocyst development was 3.4% (36/1070). Embryo transfers were performed from 17/20 nuclear transfer replicates (85%) and 3/17 recipients became pregnant (17.6%). One pregnancy aborted early (45 d), one pregnancy aborted late (126 d) and one recipient delivered a single lamb. These results show in vitro conditions for sheep oocyte maturation, NT embryo construction and embryo culture to blastocyst can be used in a nuclear transfer program to produce sheep.

	Ca containing media	**Ca Free media**
Enucleation	0/10 in "classical" metaphase	All in classical metaphase
Fusion	83/124 (62%)	81/114 (72%)
Blastocyst Development	5.5%	3.1%

Summary of NTs

CELL STAGE	**NTs Done**	**Development Rate**	**Reps Done**	**Blasts**	**Number for transfer/ reps done**
Confluent cells, late NTs	1273	3/1273 (0.24%)	13	3	3/13 (23%)
Confluent cells, late NTs, Ca free enucleation	167	0/169	4	0	0/4 (0%)
Confluent cells, early NTs	430	9/430 (2.1%)	6	9	5/6 (83%)
Confluent cells, early NTs, Ca free enucleation	487	16/487 (3.3%)	9	16	8/9 (90%)
Confluent cells, early NTs, Ca free enucleation and fusion	251	8/251 (3.2%)	6	8	5/6 (83%)

A Web-Based Reproduction Laboratory: Effect Of Web Design On Student Use And Learning

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ABSTRACT- Laboratory courses are being eliminated in the college curriculum due to inadequate faculty time, reduced funding for teaching assistants (TA), and constrained teaching budgets. Web-based technologies potentially provide the means to conserve scarce human resources for continued teaching of these laboratory courses. We have restructured a reproduction laboratory to focus on Web-based delivery of introductory material, instructions to complete the lab that include color pictures and video clips, as well as a series of questions designed to increase student understanding of the topic. The design of the graphical elements, their integration within the site and navigation through the site could affect user acceptance and student learning. We designed anatomy and histology laboratories with the graphical elements arranged in 2 different formats. The first (linear) had the graphical elements placed within the text and all aspects of the lab within one long Web page. The second (window) had the graphical elements placed on a new window that provided only the information needed to complete a single step at a time. Laboratory sections were alternated between the approaches, with students (n=42) evaluated for learning and a survey taken to ascertain student acceptance and preferences. There was no difference in the amount of interaction with the TA required (scale 1-5, with 1=none, 5=extensive) for the linear (3.00.14) or windows (2.93.09) versions. The clarity (scale 1-3, with 1=able to follow, 3=totally confusing) in the windows versions was slightly more confusing for students to follow (1.29.1 vs. 1.10.05). Students ranked the linear design better for: 1) understandability, 2) ease of navigation, 3) ease of finding information and 4) retention of information. Despite these results, there was better student learning with the window version (quiz scores, maximum=5, linear=4.0 vs. windows=4.29). Further evaluation of student learning gains is needed to confirm the trends seen in this data. Graphical design effects student learning and acceptance of web sites used for teaching purposes. Supported by USDA Higher Education Challenge Grant 2001-38411-10741.

The Common Marmoset Expresses A Fetal Zone At Birth But Not A Zona Reticularis In Adulthood.

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C19 steroid biosynthesis by the human adrenal provides an important source of androgens in the adult and fetus. Not all species rely on adrenal C19 steroid production as adults, and the placenta is a fetal source in some species. Regardless of the tissue, a hallmark of C19 biosynthetic capacity is a requirement for NADPH cytochrome P450 reductase (reductase) combined with a high P450c17:3BHSD ratio and high levels of cytochrome b5 (b5). The newborn marmoset has been reported to produce large amounts of DHEAS that fall to low/undetectable levels in adults, and raises the question of the presence of a fetal zone in newborns and a zona reticularis (ZR) in adults. We have used immunohistochemistry to stain adrenals from 1 day-old (n=2) and 1 year-old (n=4) marmosets for the enzymes b5, reductase, P450c17 and 3BHSD. Expression of b5 in 1 day-olds was undetectable in the outer 50% but strongly stained in the inner 50% of the cortex, clearly indicating the presence of a substantial fetal zone (FZ). Reductase stained slightly in the outer 50% and strongly throughout the FZ where b5 was highest. P450c17 expression showed a negative, thin outer region (zona glomerulosa/ZG) and a slightly positive middle region (ZF). P450c17 was positive throughout the FZ. 3BHSD staining was positive throughout the ZG and ZF and undetectable in the FZ. In both groups, normal serum stained negatively in the entire adrenal gland. We concluded that the newborn marmoset adrenal contains a significant fetal zone optimal for C19 steroid production. Comparative staining of 1 year-old adrenals showed undetectable staining for b5 throughout the cortex, consistent with the loss of the FZ. Reductase stained slightly positive in the ZG and more positively in the ZF. Staining for P450c17 was undetectable in the ZG and positive in the ZF. Expression of 3BHSD was strongly positive in the ZG and positive in the ZF to the medullary interface. These results are consistent with a ZG and ZF, but no ZR optimized for C19 steroid production in the adult marmoset. In summary these results are the first to clearly show that the marmoset fetal adrenal expresses a fetal zone during development similar to that in rhesus and humans but that marmosets lack a distinct ZR in adulthood. Funded by NIH grants MH60728, HL 64601, HL 56702 and HD 36913.
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Ultrastructural Features Of Bovine Granulosa Cells In A Culture Media Supplemented With Polyvinyl Alcohol (PVA)

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Our group of work has settled up an *in vitro* granulosa cell (GC) model which permits a detailed examination of the actions of specific hormones and factors that participate on follicular development and steroidogenesis. Culture systems that fail to preserve normal CG morphology may have limited physiological relevance. The main aim of the present study was to describe the intra and inter cellular structures of these GC that produce 17-β estradiol under chemically defined conditions *in vitro.* **METHODS:** Culture Method: In order to isolate GC, bovine ovarian follicles (3-5mm diameter) were dissected according to their vascularization and follicular fluid condition (clear), bisected and rinsed with Pasteur pipette with all-dressed medium: α-MEM (minimum essential medium-Gibco BRL); sodium bicarbonate; Hepes; antibiotics; selenium; transferrin; androstenedione (10^{-7}M); insulin; human rIGF-I (recombinant insulin-like growth factor); nonessential amino acids; 0.1% polyvinyl alcohol. The GC were plated at the rate of 1 x 10^6 viable cells/well. The culture was maintained at 37.5ºC with 5% CO_2 for 96 h in 24 well tissue culture plates, 70% of the medium was replaced at 48 h. Ultrastructural studies: After 24 h, 48 h, and 96 h of culture, the medium was completely removed from the wells and the cells were washed twice with PBS, and then, were washed again with cacodylate buffer (0.1M), pH 7.4. Cells were fixed in 2% glutaraldehyde + 2% paraformaldehyde in cacodylate buffer, containing 0.05% $CaCl_2$ for 2 h. Cells were postfixed in 2% OsO_4, rinsed in distilled water, dehydrated through graded series of ethanols, rinsed in acetone, embedded in Embed 812 and observed with a Phillips EM 208 electron microscope. **RESULTS:** The majority of the cells had a polyhedral shape with a low cytoplasm to nucleus ratio. The cytoplasm was abundant in organells and many mitochondria were observed. The rough endoplasmic reticulum was well developed and lipid droplets were present. Tight junctions were seen between cells. **CONCLUSIONS:** These GC cultivated under chemically defined conditions were able to maintain morphological structures related to steroid hormone synthesis, as described on previous *in vitro* and *in vivo* studies. Thus it is concluded that these cells appear to be healthy and differentiated granulosa cells.

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Embryos/Ova Recovery Rates Using Deep Or Shallow Flushing Techniques In Holstein Heifers Treated With Estradiol Benzoate Prior To Superovulation.

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This study compared recovery of embryos/ova from superovulated Holstein heifers using deep vs. shallow uterine flushing. A secondary objective was to evaluate the hormonal and superovulatory response to estradiol benzoate (EB) treatment prior to superovulation. Ten Holstein heifers (12 to 16 mo) underwent 2 superovulatory treatments in a cross-over design. Heifers were treated with decreasing doses of FSH from Day 8 to 12.5 of a synchronized estrous cycle. At 4 d prior to superovulation, half of the heifers received EB (5 mg, im) or served as Controls, followed by the alternative treatment in the subsequent superovulation. At embryo recovery, one uterine horn was flushed with shallow (~ 7 cm caudal to the tip of the horn) and the other with deep (~ 5 cm cranial to the beginning of the uterine bifurcation) flushing techniques. Embryos/ova were recovered, counted, and scored. Number of ovulations was estimated by ultrasound. Pretreatment with EB reduced circulating FSH and regressed the first wave dominant follicle with no change in number of large follicles, number of ovulations, number of embryos/ova recovered, or number of transferable embryos. The shallow flushing technique was superior to the deep technique for number of embryos/ova recovered per horn (5.4 ± 1.1 vs. 3.9 ± 0.8) or percentage of embryos/ova recovered per CL (63.9 ± 8.6% vs. 37.4 ± 6.5%). Thus, flushing the entire uterine horn increased recovery of embryos/ova.

Quality Control of PCR Products for DNA Array Production by Real-Time PCR

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DNA arrays are a useful technique for analysis of hundreds or thousands on mRNA's simultaneously. DNA fragments used for array production are frequently produced by PCR amplification of plasmid inserts. As a quality control step, gel electrophoresis analysis is usually performed to ensure adequate DNA concentration and appropriate amplification. Recently, we developed a method of assessing the quality of amplified DNA fragments using real-time PCR and melting curve analysis. DNA amplifications were performed in the presence of SYBR Green, which fluoresces in the presence of double stranded DNA. Increase in fluorescence with each cycle of amplification was indicative of DNA amplification. Fluorescence intensity of less than 1.5 relative units was not detectable by PAGE analysis followed by ethidium bromide staining. Those samples also had less than 20 ng DNA (by Hoechst 33258 dye binding) and were unacceptable for array production. When amplification was completed, a melting curve analysis was performed and derivative of fluorescence plotted against temperature to estimate melting point. Appropriately amplified fragments (those that gave a single, well-defined band on PAGE analysis and had >20 ng DNA) also had a single sharp melting point with a large derivative value (dFluorescence/dTemperature >1.6). Presence of multiple peaks, no peaks, low fluorescence or low dFluorescence/dTemperature were indicative of multiple bands or no amplification. This method provides a rapid and easily automated way to assess quality of hundreds or thousands of DNA fragments prior to array production. The only major caveat is that removal of residual SYBR Green dye should be verified prior to using arrays.

Differentiation of Endothelial Precursors from Human Embryonic Stem (ES) Cells.

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Differentiation of endothelial cells in vitro from mouse ES cells has provided important tools for our understanding of vascular formation and growth. However, the reports on development of endothelial cells differentiated from human ES cells is limited. The angiogenic factor, VEGF is required for vasculogenesis and angiogenesis during early embryonic development and plays a critical role in regulating early endothelial differentiation. In this study, using well-characterized human ES cell lines (NIH Human ES Cell Registry Code: WA01 or WA09), we will determine if a mixture of growth factors including VEGF and bFGF promote differentiation of endothelial cells from human ES cells. Human ES cells were cultured for 4 days to induce embryoid bodies (EBs) formation. After enzymatic dispersion, the EBs were cultured in differentiation media containing growth factors for 30 days. Additional EBs were treated with the same differentiated media without enzymatic dispersion for 5 days. The expression of VEGF receptor-1 (Flk-1), CD_{31} and vascular endothelial cadherin (VEcad) (three endothelial cell markers) in differentiated cells were examined using immunofluorescent staining. NIH 3T3 cells were served as a positive control for Flk-1 and negative control for CD_{31} and VEcad. Human umbilical vein endothelial (HUVE; ATCC) cells were also used as a positive control for all three antigens. Flk-1 was observed in the majority of 3T3, HUVE, and differentiated human ES cells. CD_{31} and VEcad were present in many HUVE cells and some human ES differentiated cells, but not 3T3 cells. No staining was observed in IgG controls. For the treated EBs without enzymatic dispersion, positive staining for all three antigens were localized predominantly in the center of EBs, whereas some periphery cells also exhibited positive staining. Thus, using two approaches, we have successfully induced differentiation of endothelial precursors from human ES cells. Currently, we are in the process to expand and purify these differentiated endothelial precursors, which may provide us with highly purified and unlimited endothelial cell supply for clinic applications such as tissue transplantation therapies and as a vehicle for gene therapies.

Fertilization Rate And Embryo Quality In Superovulated Holstein Heifers Inseminated With Combined Semen From Four High Fertility Bulls Or Commercial Semen.

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The present study evaluated differences in fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with combined semen from 4 high fertility bulls or commercial semen. The study also evaluated the superovulatory response to 2 different doses of FSH. Twenty heifers (12 to 16 mo) underwent 2 superovulatory treatments. Heifers were treated with decreasing doses of FSH for 4 d receiving a total equivalent of either 200 or 400 mg of NIH-FSH-P1 in a cross-over design. Ovulation was induced with GnRH 36 h after the last FSH injection and AI was performed with frozen-thawed commercial semen or combined semen from 4 high fertility bulls 12 h after the GnRH injection. Embryos/ova were recovered 7 d after GnRH, counted, and graded. Number of ovulations was estimated by ultrasound. Treatment effects were compared using t-test analysis. Fertilization rate (88.4 ± 7.6% vs. 81.9 ± 8.3%), and number of transferable embryos (3.0 ± 0.6 vs. 3.4 ± 0.9) recovered per flush for high fertility semen and commercial semen, respectively, did not differ ($P > 0.10$). The higher dose of FSH produced more ovulations per heifer (10.9 ± 1.4 vs. 7.1 ± 1.1; $P < 0.05$), but reduced fertilization rate (80.7 ± 8.8% vs. 92.2 ± 3.2%; $P < 0.05$). Consequently, the number of transferable embryos recovered per heifer did not differ (2.8 ± 0.6 vs. 3.9 ± 1.0; $P > 0.10$). Although numerically different, the percentage of transferable embryos recovered per heifer did not differ between heifers receiving the high and low doses of FSH (49.0 ± 10.0% vs. 64.0 ± 13.2%; $P > 0.10$). In conclusion, commercial semen induced a comparable high fertilization rate as the high fertility semen in superovulated heifers. Decreasing the dose of FSH to 50% of the recommended dose reduced the number of ovulations but did not reduce the number or percentage of transferable embryos recovered per heifer.

The Anti-Inflammatory Effects Of Estrogen In BV-2 Murine Microglial Cells Are Mediated By Estrogen Receptor Beta

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Estrogen exerts potent anti-inflammatory effects both centrally and peripherally. Microglia are estrogen-responsive, immunocompetent, macrophage-like cells that are the first cell type in the brain to respond to injury or infection. Microglial cell hyperactivation and their production of neurotoxic agents have been implicated in numerous neurodegenerative and inflammatory disorders, the pathologies of many of which have also been linked to the actions of estrogen. Among the agents that control microglial cell activation *in situ* are adenine nucleotides, such as ATP. ATP is found at high local concentrations in the inflammatory microenvironment, due primarily to its release from dying cells. ATP exerts many of its effects on microglia via interactions with the P2X7 purinergic receptor, a ligand-gated cation channel. To test the hypothesis that estrogen exerts anti-inflammatory effects in the brain by modulating microglial cell expression/production of molecules involved in the inflammatory response, the murine microglial cell line BV-2 was utilized. Co-treatment of BV-2 cells with estrogen and the potent microglial cell activator bacterial lipopolysaccharide (LPS) results in a dose-dependent decrease in LPS-stimulated nitric oxide production. Furthermore, P2X7 receptor protein levels were found to be up regulated in estrogen treated BV-2 cells. This result is consistent with the observation that estrogen potentiates ATP-stimulated microglial cell death. RT-PCR studies revealed that only ERβ mRNA was detectable in BV-2 cells. The ability of estrogen to promote activation of enzymes important in controlling the production of these inflammatory molecules was also investigated. Estrogen treatment of BV-2 microglia results in the rapid and sustained activation of the p38 mitogen activated protein kinase (MAPK), however, activation of the p42/p44 MAPKs, was not observed. Together, these data suggest that estrogen, via interactions with ERβ, may exert anti-inflammatory effects in the brain by modulating microglial cell expression of molecules involved in the inflammatory response, such as the P2X7 receptor and the production of nitric oxide. Comparatively little is known about the effects of ERβ in immune cells, and these results provide evidence for both latent and rapid effects of ERβ in microglial cells of the brain.

Epidermal Growth Factor Receptor and Caveolin-1 Phosphorylation

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Caveolin-1 has been shown to be necessary in cholesterol metabolism at the plasma membrane, as well as in sequestering signaling molecules in their inactive state in the membrane microdomains caveolae. Moreover, alterations in caveolin-1 expression and phosphorylation has been linked to carcinogenesis. In this regard, phosphorylation of caveolin-1 occurs in response to the activation of a number of signaling pathways, including epidermal growth factor (EGF) receptor-related pathways, although the mechanism by which caveolin-1 is phosphorylated has not yet been fully elucidated. Recently, in A431 human epidermiod carcinoma cells, EGF receptor action was shown to involve the tyrosine kinase Src in terms of mediating the phosphorylation of caveolin-1. Furthermore, caveolin-1 has been shown to be phosphorylated in A431 cells following plasma membrane cholesterol depletion and caveolae disruption by ß-methyl-cyclodextrin. To begin to test the hypothesis that caveolin-1 phosphorylation in response to cyclodextrin treatment occurs in an EGF receptor-dependent manner, we evaluated the activation of the EGF receptor and the phosphorylation of caveolin-1 in response to treatment with ß-methyl-cyclodextrin. We observed phosphorylation of the EGF receptor at tyrosine 845, which is a Src specific phosphorylation site, in response to ß-methyl-cyclodextrin treatment. These preliminary data are consistent with the concept that phosphorylation of caveolin-1 in response to ß-methyl-cyclodextrin treatment involves EGF receptor activation and the participation of a Src-related mechanism. Future studies will further test this hypothesis by evaluating the influence of EGF receptor and Src inactivation on the capacity of caveolae disruption by ß-methyl-cyclodextrin treatment to promote caveolin-1 phosphorylation. Knowledge concerning the mechanisms associated with EGF receptors and Src-dependent caveolin-1 phosphorylation should facilitate our understanding of how EGF receptor and Src cooperate in cell growth control and transformation.