Plasma folate binding capacity of the reproducing pig

Deborah L. O'Connor and Mary Frances Picciano

Division of Applied Human Nutrition, University of Guelph, Guelph, Ontario, Canada and Department of Nutrition, Pennsylvania State University, University Park, PA USA

Altered folate utilization can be a secondary manifestation of iron (Fe) deficiency during reproduction. The purpose of the present investigation was to determine whether species differences with regard to high affinity plasma folate binders (HAFBP) makes the pig an inappropriate animal model for studying the interaction of Fe and folate in the human. Specifically, we assessed the impact of gestation, lactation, Fe, and folate status on the concentration and percent saturation of HAFBP in the pig. Reproducing pigs (sows) (n = 18) were fed diets containing 1360 nmol/kg (0.6 mg/kg) folate and either 0.45 mmol/kg (25mg/kg; −) or 2.24 mmol/kg (125 mg/kg; +) Fe throughout gestation and lactation. Total folate binding capacity (TFBC) of plasma remained constant throughout gestation and lactation. Still, the mean TFBC of Fe+ sows was approximately four times that of mean plasma folate concentration. Unsaturated folate binding capacities (UFBC) of plasma were inversely correlated with plasma and red blood cell (RBC) folate values (r = −0.57 and −0.62). Both TFBC and percent saturation of HAFBP were positively correlated with indices of folate nutriture. Mean folate values of serum samples treated to remove unbound folate (23.4 nmol/L ± 2.8) did not differ from those of untreated samples (24.1 ± 2.0). Conversely, in the human the TFBC of plasma is 30 times less than plasma folate content and is not correlated with indices of folate nutriture and increases with gestation. Mean UFBC of Fe− sows was 28% greater than Fe+ sows, reflecting the lower plasma folate values among Fe− sows (P = 0.0002). Only at day 56 of gestation was TFBC of Fe− sows less than Fe+ sows (26%, P < 0.05). Overall, pig TFBC did not appear to be altered by Fe nutrition, rather it changed in response to folate nutriture. In sum, differences between the pig and the human exist with regard to HAFBP, possibly limiting the usefulness of the pig for studying folate metabolism of humans. These differences, in addition to the fact that plasma folate values among Fe− sows, like those of Fe-deficient humans, are lower than their Fe+ counterparts, suggest that impaired cellular delivery of folate is not responsible for folate depletion secondary to Fe deficiency.

Keywords: folate binding proteins; swine; folate; iron; gestation; lactation

Introduction

The nutritional requirements for iron and folate are greatest during reproduction and development, and the occurrence of iron and folic deficiencies is likewise greatest during these stages of the life cycle.[1,2] While these two nutrient deficiencies often occur concomitantly, it is generally assumed they develop independently; however, several lines of inquiry suggest that iron deficiency can predispose and even be responsible for the development of a secondary folate deficiency, especially during reproduction.[3-10] Rat pups nursed by iron-deficient dams exhibit signs of folate deficiency by 17–18 days of age.[6-9] Similarly, rapidly growing piglets nursed by sows fed diets low in iron 0.45 mmol/kg (25 mg/kg) throughout gestation and lactation are depleted of folate by 21 days of age.[10]

Depressed milk folate secretion is an early manifestation of iron deficiency in the rat dam and is thought to be at least partially responsible for folate depletion in their nursing pups.[7-9] It is unknown whether iron-deficient pups are able to compensate for low milk folate by increasing absorption of dietary folate or whether neonatal iron deficiency impairs folate absorption. Due to docu-

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Address reprint requests to Dr. D.L. O'Connor at the Division of Applied Human Nutrition, University of Guelph, Guelph, Ontario, Canada N1G 2W1.
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mented differences in folate absorption between humans and rats, we continue to investigate the feasibility of using maternal and neonatal swine as models for studying the interaction of iron and folate in human beings.

A number of similarities between swine and humans exist with regard to folate absorption and iron metabolism. First, the mechanism by which naturally occurring folates are absorbed is similar in the human and swine.[11-13] Second, the impact that gestation and lactation has on maternal plasma and red blood cell (RBC) folate values is remarkably similar in both species. We previously reported that by mid-gestation, sow plasma and RBC concentrations were reduced by at least 50% as compared with those values at conception.[10] These reductions are of the same order of magnitude as those reported in unsupplemented pregnant women.[14]

Third, it is clear that rats fed an iron-deficient diet through pregnancy will give birth to pups with iron deficiency anemia.[9] In contrast, the iron status of the newborn human and pig is relatively unaffected by maternal iron nutriture.[15-22] Further, milk iron concentration does not appear to be significantly influenced by maternal iron status in humans or swine.[19,23] Maternal iron deficiency in the rat results in a reduction in milk iron concentration.[24]

Despite similarities in folate absorption and iron metabolism between the human and the pig, significant differences in folate metabolism may be present. Specifically, Mantzos et al.[25] reported a significant quantity of high affinity folate-binding protein in the plasma of swine. While this avid folate binder is present in human plasma, the total binding capacity of this protein is relatively insignificant, except during pregnancy.[26-29] The concentration of high affinity folate binder in human plasma appears unrelated to folate nutrition.[26-28] Whether the quantity of high affinity folate binder in the pig increases during gestation or is related to folate nutrition is unknown. Further, it is unclear, due to mere quantity, if these avid folate binders influence the partitioning of folate between plasma and other tissues.

The purpose of the present investigation was to further assess the appropriateness of using maternal swine as a model for investigation of iron and folate metabolism of human beings. Specifically, we examined the role of high affinity plasma folate binders (HAFBP) in the interaction of Fe and folate during reproduction in the pig. Further, we assessed the impact of pregnancy and lactation on total folate binding capacity (TFBC), unsaturated folate binding capacity (UFBC), and percent saturation of folate binders in the plasma of sows. These relationships were compared with those reported in the literature for humans. In addition, we examined the relationship of these measures with indices of maternal iron and folate status.

Methods and materials

Animal care

Eighteen multiparous sows (181–239 kg) were bred and immediately assigned to one of two dietary treatments (nine sows/treatment). Consistent with the usual feeding regime of the farm, sows received 2 kg daily of the experimental diet throughout gestation and were provided with the diets ad libitum during lactation. During gestation sows were housed in aluminum crates in a controlled temperature (20–22° C) housing facility. One week prior to the expected date of parturition, sows were transferred to elevated farrowing crates equipped with plastic mesh floors, aluminum walls, and stainless steel feeders. Water was provided to the animals ad libitum throughout the study and was regularly monitored for iron content.

On day 114 of gestation, labor was induced by administering 10 mg PGF_2 analog (Lutalyse, UpJohn, Kalamazoo, MI USA). The day after delivery, litters were weighed and culled to eight pigs (four males, four females).

Experimental diets

The experimental diets were formulated to meet all known nutritional requirements for the reproducing sow except iron.[30,31] A detailed description of the diets can be found in a previous publication.[10] Individual diet constituents were selected on the basis that they contained low levels of endogenous iron (Fe). A common basal diet was prepared without added Fe and one-half of this mixture was supplemented with 1.79 mmol/kg (100 mg/kg) of Fe in the form of $FeSO_4 \cdot 7H_2O$. Direct analyses confirmed dietary iron contents of 0.45 mmol/kg (Fe – , 0 mmol/kg supplemental Fe) or 2.24 mmol/kg (Fe + , 1.79 mmol/kg supplemental Fe).

Blood collection

On days 1, 56, and 109 of gestation (± 3 days) and day 7 and 21 of lactation, approximately 10 mL of blood was collected from the anterior vena cava of sows using heparinized needles and syringes. Aliquots (100 µL) of the whole blood were taken and diluted with 9 volumes of 0.05 M sodium phosphate buffer (pH 7.0) containing 1–2% ascorbate. After aliquots of whole blood were removed for determination of hematocrit and hemoglobin concentration, remaining whole blood was centrifuged, and the plasma was separated and stored with 1% ascorbate. Blood samples were immediately frozen and stored at – 70° C until analyzed.

Folate analyses

The folate concentration of plasma, red blood cells, and diets were determined microbiologically using *Lactobacillus casei* (ATCC 7469) according to the protocol described by Keagy[32] with slight modification. Briefly, thawed plasma and buffered lysed blood samples were incubated 15 minutes (room temperature) to permit plasma folate conjugase to free folate from its microbiologically non-assayable form. Diet samples were mixed with 9 volumes of 0.1 M phosphate buffer (pH 7.0) containing 5 mg/L ascorbate. The mixture was then homogenized, heated for 5 minutes (121° C), and centrifuged. Total folate concentration in the resultant supernatant was determined following an 8-hour incubation (37° C) with partially purified folate conjugase prepared from chicken pancreas acetone powder (Sigma Chemicals, St. Louis, MO USA).[33]

Plasma folate binding characteristics

The unsaturated folate binding capacity (UFBC) and TFBC of pig plasma was determined by the method of Coleman and Herbert[27] with minor modification to accommodate the anticipated increase in folate binding. UFBC was determined by mixing swine plasma with 1 M potassium phosphate buffer (pH 7.4) and ^{3}H-PteGlu (34 Ci/mmol folic acid) (Amersham Canada Ltd., Oakville, Ontario, Canada). Following a 15-minute incubation at room temperature, unbound folates (la-

beled and endogenous) were removed by addition of a hemoglobin-coated charcoal solution (Hbcc). Tubes were mixed, centrifuged (10 minutes), and the resultant supernatant transferred to liquid scintillation cocktail (ScintiVerse, Fisher Scientific, Unionville, Ontario, Canada).

To determine the TFBC of swine plasma, endogenous folates were disassociated from binding proteins by incubating plasma with 0.5 mL of an acidified potassium phosphate buffer (pH < 4) at room temperature for 15 minutes. Folates in the mixture were removed by addition of Hbcc as described above. The resultant supernatant was transferred to a tube containing potassium phosphate buffer and NaOH to bring the pH of the mixture back to 7.4. Total folate binding capacity was then measured as described above for UFBC. The percentage saturation of folate binding proteins was calculated as shown in Equation 1.

$$\frac{(\text{TFBC} - \text{UFBC})}{\text{TFBC}} \times 100 \qquad (1)$$

A quench curve consisting of measured amounts of C_2Cl_4 and a constant amount of ^{3}H-PteGlu was simultaneously prepared. Data were corrected for background and non-specific binding. To assess the reproducibility of results, pooled human and swine plasma samples were performed with each assay.

Iron analyses

Hematocrits and hemoglobins were measured on freshly collected blood. Hemoglobin concentrations were determined by the cyanomethemoglobin method[34] using prepared reagents and standards (Sigma Chemicals). Diets were analyzed for Fe using atomic absorption spectrophotometry (Thermo-Jarrell Ash Model 951, Franklin, MA USA). The accuracy of this analytical procedure was assessed using orchard leaves no. 1571 as the standard reference material (National Bureau of Standards, Gaithersburg, MD USA).

Statistical analyses

Data were evaluated by repeated measures analysis of variance using the Statistical Analyses System (Cary, NC USA).[35,36] Where appropriate, differences between Fe treatments at each time point were determined by least significant difference tests. A probability level of 5% was chosen as the level of statistical significance. Pearson correlation coefficients were computed to assess the strength of association between indices of Fe and folate status with that of UFBC, TFBC, and percent saturation of folate-binding proteins.

Results

Maternal feed intake, body weights and iron status

Dietary iron concentration had no effect on food consumption or the body weights of sows during reproduction (data now shown).[10] Wide pig-to-pig variation existed among indices of iron status. Despite this, maternal dietary iron concentration was shown to significantly influence mean sow hematocrit and hemoglobin concentrations (*Table 1*). In general, hemoglobin concentrations among sows fed 0.45 mmol iron/kg diet (Fe −) were lower than those of sows fed 2.24 mmol iron/kg (Fe +) ($P < 0.05$). On day 109 of gestation, Fe − sows had a mean hematocrit value 16% lower than that of Fe + sows ($P < 0.05$). While Fe − sows continued to have lower mean hematocrit concentrations on days 7 and 21 of lactation, these differences were not statistically different from Fe + sows ($P < 0.06$–0.07).

Folate status

Regardless of dietary treatment, sows in the present study showed evidence of rapid folate depletion during gestation (*Table 1*). By mid-gestation, mean folate concentrations of plasma and RBCs were reduced by at least 50% compared with values obtained at breeding ($P < 0.0001$). Further, the absolute quantity of folate found in RBCs was very small. The folate content of RBCs was at best only three times that of plasma, and by day 21 of lactation mean RBC folate concentrations did not significantly differ from that of plasma. Despite a significantly greater mean plasma folate concentration among Fe − sows on day 1 of gestation, their mean plasma folate concentrations were reduced by 53% and 31% compared with Fe + sows on day 7 and 21 of lactation, respectively.

Plasma folate binding characteristics

After breeding, the UFBC of sow plasma significantly increased ($P < 0.0001$), reflecting the decrease in circulating folate concentration (*Figure 1*). UFBC were inversely correlated with plasma ($r = -0.62$) and RBC (-0.57) folate concentrations ($P = 0.0001$). While TFBC was positively correlated with plasma folate concentrations ($r = 0.42$, $P = 0.004$), no significant correlation existed between TFBC and RBC folate concentrations ($r = 0.23$, $P = 0.1358$). As would be expected, the percentage of folate binding sites occupied by folate was positively correlated with plasma ($r = 0.74$), and RBC ($r = 0.66$) folate concentrations ($P = 0.0001$).

Maternal dietary iron concentration did influence plasma folate values and plasma folate binding characteristics of sows. Despite significantly greater mean values for plasma folate concentration, and percent saturation of HAFBP at breeding, mean values for these parameters were significantly reduced among Fe − sows compared with Fe + sows at subsequent sampling times. On day 56 of gestation, the mean percent saturation of HAFBP was reduced by 47% compared with that of Fe + sows. As a group, Fe − sows had greater UFBCs than their Fe + counterparts ($P = 0.0002$).

Discussion

Results from the present study suggest that differences between the human and pig exist with regard to HAFBP. The high absolute concentration of HAFBP in the pig suggests that these binders may have a greater impact on the folate metabolism of the pig compared with that of the human. The absolute concentration of HAFBP in the pig is approximately 400 times that of the human. The mean TFBC of control sow plasma on days 1, 56, and 109 of gestation and on days 7 and 21 of lactation was 146.7, 138.9, 132.3, 151.2, and 144.2 nmol/L, respectively. In sharp contrast, Colman and Herbert[27] and Corrocher et al.[28] report mean TFBC val-

Table 1 Mean indices of iron and folate status of sows receiving two levels of dietary iron during a single reproductive cycle[1,2]

	Dietary Fe (mmol/kg)	Day of Gestation 1	Day of Gestation 56	Day of Gestation 109	Day of Lactation 7	Day of Lactation 21
Hematocrit[a,b,c]	2.24	31.6 ± 0.8	37.7 ± 0.8	38.5 ± 0.9	37.0 ± 0.6	38.1 ± 1.2
%	0.45	30.6 ± 1.4	38.8 ± 1.2	32.2 ± 2.5*	34.0 ± 1.4	35.0 ± 1.4
Hemoglobin[a,b]	2.24	119 ± 4	125 ± 4	132 ± 3	120 ± 2	123 ± 5
(g/L)	0.45	119 ± 5	124 ± 4	121 ± 3*	113 ± 5	118 ± 4
Plasma folate[b,c]	2.24	63.4 ± 5.0	35.6 ± 2.7	24.9 ± 3.2	25.4 ± 0.9	33.1 ± 3.6
(nmol/L)	0.45	83.4 ± 15.9*	27.2 ± 8.4	23.6 ± 7.9	12.0 ± 3.4*	22.7 ± 8.8*
RBC folate (nmol/L)[b]	2.24	178.6 ± 39.9	85.7 ± 13.8	56.9 ± 10.2	62.6 ± 20.2	46.2 ± 14.7
(packed RBCs)	0.45	198.3 ± 39.2	59.4 ± 22.4	48.0 ± 17.0	39.2 ± 15.0	24.0 ± 13.8
% Folate binding	2.24	71.7 ± 10.0	60.1 ± 9.9	38.2 ± 6.4	39.3 ± 2.8	37.9 ± 9.0
Saturation[a,b]	0.45	66.1 ± 10.5	31.0 ± 20.3	33.3 ± 6.4	22.4 ± 6.3	26.4 ± 15.7

[1]Values are means for 5–7 sows ± SEM.
[2]Repeated analysis of variance (2 × 5).
[a]Significant effect of dietary iron concentration ($P < 0.05$).
[b]Significant effect of reproductive stage ($P < 0.05$–0.001).
[c]Significant interaction between dietary iron and stage of reproduction.
*Significant differences between dietary treatment groups at a particular time point ($P < 0.05$).

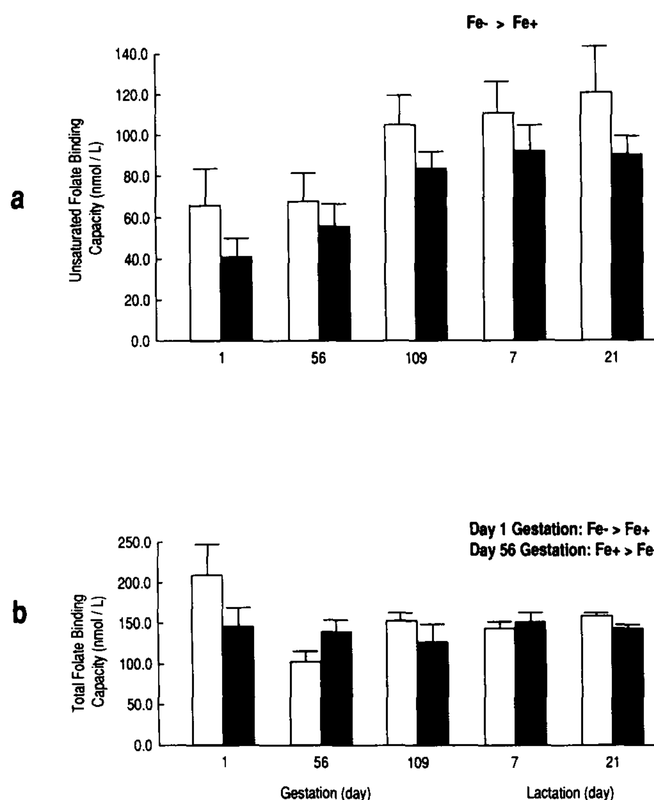

Figure 1 Unsaturated and total folate binding capacity (mean ± SEM) on days 1, 56, 109 of gestation and days 7 and 21 of lactation for sows fed 1360 nmol folate/kg diet and either 0.45 mmol/kg (Fe−) (open bars) or 2.24 mmol iron/kg diet (Fe+) (solid bars). Repeated analysis of variance revealed significant dietary iron and temporal effects on unsaturated folate binding capacity (a). Further analysis revealed a significant dietary iron, temporal interaction on total folate binding capacity (b).

ues of 0.3943 and 0.3422 nmol/L, respectively, among non-pregnant humans. These two groups also report mean TFBC values of 0.5824 and 0.5615 nmol/L during the third trimester of pregnancy.

Second, not only do large differences in absolute concentration of TFBC exist between these species, significant differences exist with regard to the ratio of these binders to serum folate concentration. Colman and Herbert report that the concentration of serum folate is approximately 30 times that of the TFBC in humans.[27] Conversely, the folate concentration of sow plasma in the present study was less than the TFBC of plasma. Mean plasma folate concentrations among control sows on days 1, 56, and 109 of gestation and days 7 and 21 of lactation were 43, 26, 19, 8, and 23% that of the TFBC, respectively. To directly ascertain the percentage of plasma folate bound to these HAFBP, a separate experiment was conducted in which serum folate content was determined microbiologically with and without prior Hbcc treatment of samples. The mean serum folate concentration of samples ($n = 20$) treated with charcoal to remove unbound folate (23.4 nmol/L ± 2.8) (mean ± SEM) did not differ from those samples not treated with Hbcc (24.1 ± 2.0), indicating that the majority of folate is bound to HAFBP in pig serum.

Third, UFBC, TFBC, and percent saturation of HAFBP in sows, regardless of iron nutriture, were found to be correlated with indices of folate nutriture. While unsaturated folate binding capacity was inversely correlated with plasma ($r = -0.62$, $P = 0.0001$) and RBC ($r = -0.57$, $P = 0.0001$) folate concentrations, TFBC was positively associated with plasma folate concentration ($r = 0.42$, $P = 0.004$). The percentage of high affinity binding sites occupied by folate was positively correlated with plasma ($r = 0.74$) and RBC (r

= 0.66) folate concentrations ($P = 0.0001$). In contrast, most investigators report no correlation between TFBC and UFBC and indices of folate nutriture in the human. In fact, many question the physiological role of these binders in humans.[26-28,37]

Finally, while the concentration of the HAFBP progressively increases with advancing stages of pregnancy in rats, sheep, and humans,[27-29,37,38] they do not in sows. Rather, the TFBC of sows remained relatively constant throughout gestation and lactation. UFBC does increase from day 1 to day 109 of gestation and remains elevated throughout lactation in the pig. Similarly, the mean percent saturation of HAFBP decreases from day 1 to day 109 of gestation and remains low throughout lactation.

Despite the strong correlations between blood folate values and TFBC, UFBC, and percent saturation of HAFBP, the mean folate concentration of pig plasma as determined directly by microbiological assay was consistently lower than that calculated indirectly from the difference between TFBC and UFBC. While the reasons for this discrepancy are not clear, it is unlikely that the presence of high concentrations of HAFBP in the pig interfere with the microbiological assay for folate as they were denatured by heating (120° C for 5 minutes) prior to analysis.[39] It is likely that the values calculated from the radioassay overestimate the folate content of pig plasma. Natsuhori et al. suggest that the circulating forms of folate in the pig differ from those of many species, including the human.[40] As generally accepted, these investigators found that methyltetrahydrofolate (CH_3-H_4-folate) was the predominant folate found in the plasma of rats, mice, rabbits, dogs, cows, horses, and humans. In contrast, the concentration of H_4-folate was significantly greater than the concentration of 5-CH_3-H_4-folate in the plasma of pigs as determined by high pressure liquid chromatography with an electrochemical detector. Natsuhori et al.[40] demonstrated that the radioassay tends to overestimate total folate in the plasma of pigs because of a 4-to-1 preference of H_4-folate over other folates (CH_3-H_4-folate or synthetic folic acid) for high affinity folate binders in the assay system.

While the exact metabolic function of HAFBP is not clearly defined, a number of functions have been proposed.[41-43] Like the plasma binding proteins for the transport of B_{12}, iron, and thyroid hormone, it is speculated that HAFBP may protect circulating folate from proteolytic degradation and delivery of folate to target tissues. Natsuhori et al. suggest that the high concentration of HAFBP in the pig permits the existence of significant quantities of H_4-folates, which are known to be extremely easy to oxidize.[40] Further, it has been proposed that HAFBP scavenge oxidized (inactive) circulating folates and return them to the liver for reduction and conversion to 5-CH_3-H_4-folates. There is also evidence to suggest that HAFBP trap folates in fetal circulation to facilitate fetal growth.[26,41,44,45] Other investigators question the physiological significance of HAFBP in humans. Because of the homology between HAFBP and folate binding proteins in other tissues, it is suggested that HAFBP are of tissue origin, and their presence in plasma is incidental.[46]

Mason and Selhub[47] suggest that folates bound to HAFBP may, in fact, be transported across cell membranes by a mechanism distinct from that of unbound folate and this process does not require prior dissociation of the vitamin-protein complex. Using an in vivo intestinal loop technique and suckling rats, they found that folate bound to high affinity folate binders from milk were absorbed most avidly in the ileum and that absorption was not inhibited by sulfasalazine, a folate-transport inhibitor. In contrast, unbound folate was primarily absorbed in the jejunum, and this process was inhibited by sulfasalazine. In view of the fact that folate is primarily bound to HAFBP in the pig, and considering the high concentration of these binders in the plasma of the pig relative to other species, we suggest that folate may be delivered to and taken up by cells by an endocytotic mechanism similar to that suggested by Mason and Selhub.[47]

Statistical analyses of data in the present study revealed a significant effect of dietary iron treatment on HAFBP characteristics; however, these changes appear to be mediated by changes in blood folate concentration rather than changes in iron nutrition per se. For example, Fe + sows that consumed the experimental diet for 56 days had a mean plasma folate concentration and TFBC greater than that of Fe − sows. On day 56 of gestation, Fe + sows also had significantly higher mean hematocrit and hemoglobin concentrations. The Fe + group had a significantly lower mean TFBC and plasma folate concentration than the Fe − group prior to consuming the experimental diets, despite similar iron status values, suggesting that changes in TFBC reflect changes in blood folate values rather than maternal iron nutriture. Further, no significant correlations were found between indices of iron status and TFBC.

As reported in studies examining humans and rats, iron nutrition influences folate utilization in the reproducing pig.[3-10] Sows fed 0.45 mmol/kg Fe had significantly lower mean plasma folate concentration on days 7 and 21 of lactation than sows fed 2.24 mmol/kg iron. This iron-folate interaction occurred despite the fact that the iron content of diets fed to sows had no direct effect on HAFBP and the TFBC of sow plasma in particular. These observations imply that the site responsible for folate depletion secondary to iron deficiency is not at the stage of placenta or cellular folate delivery where the human and the pig differ.

In conclusion, results from the present study suggest that significant differences between the pig and the human exist with regard to high affinity folate binders, possibly limiting the usefulness of the model for studying folate metabolism of humans. Second, TFBC is not altered by iron nutrition, rather it changes in response to altered folate nutriture, suggesting that impaired TFBC is not responsible for impaired folate utilization among sows fed low levels of iron in their diet.

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