

Title: Measurement of cord insulin and insulin related peptides suggests females are more insulin resistant than males at birth

Received for publication 17 July 2006 and accepted in revised form 26 April 2007.

Abbreviated Title: Females are more insulin resistant at birth

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Abstract:

Objective: We aimed to examine gender differences in insulin and insulin propeptide concentrations at birth using validated cord blood collection.

Research Design and Methods: 1) We tested the impact on insulin and insulin propeptides of taking 13 cord blood samples in heparin and EDTA and then centrifuging and separating plasma after 1, 2, 24, or 48 hours at room temperature (heparin) or 4°C, (EDTA). 2) Cord plasma insulin and insulin propeptides concentrations were measured in 440 babies and correlated with offspring anthropometry measured at birth.

Results: 1) Cord insulin concentrations significantly decreased (74% baseline by 24 hours, $p=0.01$) in the samples taken in heparin and stored at room temperature, but those taken on EDTA and refrigerated remained stable for up to 48 hours. Insulin propeptides were stable in both. 2) Cord plasma insulin and insulin propeptides measured in EDTA were related to all measures of birth size and maternal glycaemia and BMI ($r>0.11, p<0.03$ for all) and were higher in those delivered via Caesarean section. Females were lighter (3497 v 3608g, $p=0.01$) but had higher cord insulin (46.7 v 41.2 pmol/l, $p=0.031$), total proinsulin (34.1 v 25.8 pmol/l, $p<0.001$), and intact proinsulin (9.5 v 8.3 pmol/l, $p=0.004$) concentrations than males, a difference further confirmed when comparing girls and boys cord insulin when they were pair-matched for birth weight (insulin: 49.7 v 42.1 pmol/L, $p=0.004$).

Conclusion: When using appropriate sample collection methods, females have higher insulin concentrations than males at birth, despite being smaller, suggesting intrinsic insulin resistance in females.

Introduction:

There is increasing evidence to suggest that girls are more insulin resistant than boys. This has been shown in fasting insulin measurements, frequently sampled IV glucose tolerance tests, and euglycaemic clamps in children from five years of age (1), through late childhood (2, 3) and puberty and adolescence (4-6). The increase in insulin resistance (IR) is seen in white Caucasian, Afro-Caribbean and Asian Indian races (1-4, 6). Furthermore, Type 2 diabetes in children is far more common in females than males (7-9).

The fact that gender differences are seen early in life could reflect differences in intrinsic insulin resistance or differences in postnatal behaviour. In a study of 307 subjects, Murphy et al. (1) were unable to explain gender differences at 5 years old by looking at differences in anthropometry and physical activity. If the insulin resistance is “intrinsic” this would suggest that it is genetically determined and should be apparent from birth. An insulin resistant phenotype at birth has been previously described in Indian babies (10), who have higher umbilical cord insulin concentrations and more subcutaneous fat than UK babies, despite being lighter. If girls are more insulin resistant than boys, one might expect to observe a similar phenotype at birth, with girls having higher cord insulin concentrations, even though they are lighter than boys. Girls are consistently lighter on average than boys, however, the results of cord insulin and insulin propeptide measurements are contradictory. Higher concentrations of proinsulin and split proinsulin have been recorded in girls (11-13) but no significant gender differences in insulin were found (11-14).

One possible explanation for why gender differences in umbilical cord insulin

concentrations have not previously been seen may be due to instability of insulin in cord blood. Lindsay et al.(13) found insulin concentrations in cord blood fell rapidly to approximately 20% of baseline with a 24 hour delay before centrifugation and freezing,. In contrast, they found proinsulin remained stable in cord blood for up to 24 hours. Another explanation could be that insulin, which shows pulsatile release and has a shorter half-life than proinsulin (15), may be more susceptible to fluctuations associated with delivery. (12).

We aimed to establish a valid procedure for collecting cord blood for insulin assay, then to use this to assess differences and inter-relationships between insulin, intact proinsulin and total proinsulin, and to examine whether there are any gender differences in insulin and insulin propeptides at birth.

Research Design and Methods:

Ethical approval was given by the North and East Devon Local Research Ethics Committee and informed consent was obtained from the parents of the newborns.

Study 1 – Stability of insulin and insulin propeptides in cord blood

13 non diabetic women gave consent for the collection of blood from the umbilical cord after delivery.

A 20ml sample of blood was taken from the umbilical cord vein immediately following delivery of the placenta. This was transferred to 5 lithium heparin tubes and 5 potassium EDTA tubes (non-gel tubes, Sarstedt, Leicester UK). One heparin and one EDTA sample were centrifuged and the plasma separated and frozen at -80°C immediately. The remaining 4 heparin samples were stored at room temperature (as described by Lindsay et al. (13)), and the remaining 4

EDTA samples were stored at 4°C. These samples were then centrifuged and frozen after 1, 2, 24, and 48 hours.

All samples were stored at -80°C for less than a month before being sent on dry ice to the Regional Endocrine Laboratories in Birmingham where insulin, total proinsulins and intact proinsulin assays were performed.

Plasma insulin, total proinsulins and intact proinsulin were measured by immunochemiluminometric assays (ICMA) (Molecular Light Technology, Cardiff, UK). The insulin assay was specific for insulin with a quoted interassay imprecision (Coefficient of variation <10%) over the range 6.5-169 pmol/L, and quoted cross-reactivities of 1.2% for intact proinsulin, 1.6% for des 31-32 split proinsulin, and 44% for des 64-65 split proinsulin.

For the assays for total proinsulins and intact proinsulin the quoted interassay imprecision (CV < 10%) was 3.0-257 pmol/L and 8.9-390 pmol/L, respectively. Quoted cross-reactivity for insulin in the total proinsulin assay was 2% and was not detectable in the intact proinsulin assay.

All results were analysed using non-parametric statistics due to the small numbers. The Mann-Whitney U test was used to assess differences between results at baseline and subsequent time points.

Study 2 – Inter-relationships of insulin and insulin propeptides

Recruitment and protocol

Families were recruited as part of the Exeter Family Study of Childhood Health, a large prospective study examining genetic influences on fetal and early growth (16). Maternal weight, height, BMI and fasting glucose and insulin concentrations were measured at 28 weeks gestation. Maternal and cord plasma glucose were assayed by the pathology

laboratories at the Royal Devon and Exeter Hospital, Exeter, using a standard laboratory method (coefficient of variation <2%). Maternal insulin was measured using the same assay as before. Insulin resistance in the mother was calculated using homeostasis model assessment (HOMA) program for specific insulin measurement (17, 18).

Detailed anthropometry was taken on the child at birth, including weight, length, head circumference and skinfold thickness of the tricep and subscapula.

A sample of cord blood (EDTA plasma) was taken following delivery of the placenta by the midwife on duty at the time of delivery, and stored at 4°C until the research midwife was able to centrifuge and freeze the separated plasma at -80°C (median time 11 hours). Insulin, total proinsulin and intact proinsulin were measured using the same assays as in the stability study

Statistics

Insulin and insulin propeptide results were log transformed to ensure normal distribution. Pearson correlations were used to assess relationships between insulin and proinsulin concentrations with birth size, and maternal anthropometry and biochemistry. Analysis of variance was used to test for differences between modes of delivery. T-tests were used to assess gender differences. Multiple linear regression analysis was carried out to explore differences in gender whilst accounting for potential confounders. Finally, girls and boys were pair-matched for birthweight and gestation, in a similar analysis to that carried out by Yajnik et al.(10), to further investigate gender differences in insulin and insulin-propeptides.

Results:

Study 1 – Effect of delay in centrifugation and freezing on insulin,

total proinsulin, and intact proinsulin concentrations

There were no differences in concentrations of insulin, total proinsulins or intact proinsulin between cold storage-EDTA and room temperature-heparin samples at baseline (medians: insulin: 52.6 v 51.9, $p=0.78$; total proinsulins: 30.7 v 34.9, $p=0.96$; intact proinsulin: 15.1 v 15.6; $p=1.0$).

Insulin was far less stable when blood was taken on heparin and stored at room temperature, with a reduction to 74% of baseline (IQR: 69-84%) by 24 hours ($p=0.012$) and to 49% of baseline (IQR: 36-57%) by 48 hours ($p<0.001$). This is in contrast to samples taken on EDTA and stored at 4°C, where insulin concentrations were similar (median 96 - 98% of baseline, $p>0.6$ for all) throughout the 48 hour period (Figure 1a). Time delay in centrifugation and freezing had little effect on total and intact proinsulin concentrations measured in both cold storage-EDTA and room temperature-heparin samples (Figures 1b and 1c), with most results remaining within 10% of baseline for up to 48 hours. The only deviation from this was with the intact proinsulin concentrations from the 48 hour heparin samples which decreased to 81% (IQR: 75-91%) of baseline, although this difference did not quite reach statistical significance ($p=0.06$).

Four of the heparin samples were found to be haemolysed (1 at 24 hours and 3 of the 48 hour samples). Removal of the 4 haemolysed heparin samples made little difference to the results with insulin still significantly reduced to 54.7% (IQR: 38.6-66.1%) of baseline ($p<0.001$) in samples taken on heparin and left 48 hours before centrifugation and freezing (compared to 93.5% of baseline for total proinsulin and 83.7% for intact proinsulin).

Study 2 – Inter-relationships between insulin and insulin propeptides at birth

a) Characteristics of babies and Insulin and Insulin Propeptide concentrations

Only those subjects with all three umbilical cord insulin peptide measurements (insulin, total proinsulins and intact proinsulin) were included in this study. Insulin and insulin propeptides were not measured in 92 samples due to haemolysis. The analysis was therefore carried out on 440 terms, singleton babies. These babies had a mean (SD) birthweight of 3554 (477)g and a mean (SD) gestation of 40.1 (1.2) weeks. 229 (52%) were males and 180 (41%) were primips. 63 (14%) were born via Caesarean section. Those where all 3 cord peptide results were available were slightly heavier than those with at least one of the measurements missing (3554 v 3463g, respectively, $p=0.003$), but there was no difference in gestation (40.2 v 40.1, $p=0.583$).

Cord insulin concentrations were associated with intact and total proinsulin concentrations ($r=0.446$ and 0.666 , respectively, $p<0.001$), and intact and total proinsulin were also strongly correlated ($r=0.680$, $p<0.001$). There was greater variation in the total proinsulins and insulin compared with the intact proinsulin ($F=1.6$ and 1.4 , respectively, $p<0.001$ for both).

The median total proinsulin:insulin ratio was 0.66. Total proinsulins accounted for 40.8% of all insulin-like molecules in cord blood.

b) Relationships with Mode of Delivery (Figure 2)

Babies delivered by Caesarean section had significantly higher insulin concentrations than babies delivered by normal vaginal delivery or assisted delivery (61.9 v 41.7 and 39.2 pmol/L respectively, $p<0.001$)

(Figure 2a). There was also a small but significant difference in intact proinsulin concentrations between Caesareans and normal vaginal deliveries (11.1 v 9.0 pmol/l, respectively, $p=0.03$), but there was no difference in total proinsulin concentrations between the three modes of delivery. Those born via Caesarean section also had higher birthweights than those delivered vaginally, when correcting for sex and gestation (3663 v 3505g, respectively, $p=0.009$), but even when adjusting for birthweight, those born via caesarean section still had higher insulin and intact proinsulin concentrations. Those born via emergency section had higher cord insulin concentrations than those born via elective sections (69.7 v 55.2 pmol/l, respectively) although this did not reach significance ($p=0.11$).

c) Differences between Insulin and Insulin Propeptides and their Relationships with Birth Size and Maternal Factors

Insulin, total proinsulin, and intact proinsulin were significantly correlated with all measures of birth size including weight, length, head circumference and skinfold thicknesses ($r=0.213-0.465$, $p<0.001$ for all). The strength of the correlations for each measure was similar for insulin, total proinsulin, and intact proinsulin with each being within the 95% confidence intervals of one another. As expected, fasting maternal glucose at 28 weeks was significantly associated with all three insulin peptides ($r=0.16-0.26$, $p\leq 0.001$ for all). Maternal age, weight and BMI were also significantly correlated with all three peptides ($r=0.11-0.18$, $p<0.001 - 0.03$). Maternal insulin and HOMA-R were significantly correlated with cord insulin and total proinsulin ($r=0.17-0.23$, $p<0.001$ for all), but not with intact proinsulin ($r=0.05$, $p=0.27$ and $r=0.06$, $p=0.23$ respectively). Removing data from those delivered by Caesarean sections made no difference to results.

e) Gender differences in Insulin and Insulin Propeptides (Figure 2b)

Cord plasma from females had higher concentrations of insulin (46.7 v 41.2 pmol/l, $p=0.031$), total proinsulin (34.1 v 25.8 pmol/l, $p<0.001$), and intact proinsulin (9.5 v 8.3 pmol/l, $p=0.004$) than males (see Figure 2b). Total proinsulin:insulin ratio was higher in girls than boys (0.73 v 0.63, respectively, $p=0.003$). Similarly, the percentage of total proinsulin out of the total insulin-like molecules was also higher (42.5 v 39.2%, $p=0.003$).

Multiple Linear Regression Analysis was carried out to assess the independent contribution of baby's sex when accounting for other determinants of cord insulin concentrations (gestation and maternal age, BMI, glucose and insulin). When insulin was examined, gender did not quite remain a significant independent determinant (B(SE)=0.046(0.025), $p=0.064$), but it was significantly associated with total proinsulin (B(SE)=0.106(0.026), $p<0.001$) and intact proinsulin (B(SE)=0.052(0.022), $p=0.017$).

Females were lighter (3497 v 3608g, $p=0.01$) and shorter (49.8 v 50.8, $p<0.001$) than males, but had more subcutaneous fat as measured by tricep and subscapula skinfold thicknesses (5.0 v 4.7mm, $p=0.01$ and 5.1 v 4.8cm, $p=0.01$, respectively).

Final exploration of the gender difference was carried out using pair matching where girls and boys were matched for birthweight (to nearest 100g) and gestation (within 5 days). 161 boy-girl pairs could be matched using these criteria. There was a slight bias in the matching as girls appeared to have slightly longer gestations (40.2 v 40.1 weeks, $p<0.001$). Although this difference works out at less than a day, its significance in a paired t test is probably

due to girls being smaller than boys and, therefore, to pair match on weight, the girls would generally be of a longer gestation. When matching for birthweight and gestation, girls still had higher concentrations of all 3 peptides (see Table 1).

The gender difference in cord insulin concentrations could reflect a difference in cord glucose concentrations. Cord glucose concentrations were negatively associated with time before centrifugation ($r=-0.483$, $p<0.001$). However, there was no difference in this time between girls and boys (10.3 v 11.4 hours, $p=0.521$), therefore, a relative difference in cord glucose concentrations could be assessed. There was no difference in cord glucose concentrations between girls and boys (3.5 v 3.5 mmol/l, $p=0.678$). Adjustment of cord glucoses for time before centrifugation to 0h further confirmed there was no gender difference (4.4 v 4.3, $p=0.640$).

There was no difference in the number of Caesarean sections between boys and girls (30 v 33, $p=0.45$) and exclusion of babies delivered by Caesarean section from the analyses made no difference to the results.

Conclusions:

We have provided data that shows that females have higher insulin and proinsulin concentrations and total proinsulin:insulin ratio in cord blood than males despite weighing less at birth. As insulin is a principal growth factor in utero, the higher insulin coupled with reduced growth in females suggests that females are more insulin resistant in utero as well as after birth.

The higher insulin and proinsulin(s) in girls compared to boys for glucose/weight indicate insulin resistance in girls. The higher proinsulins in girls compared to boys has been noted before in neonates (11-13) and these are the more stable

molecules. Changes in ratios and percent may not have been seen if stability of insulin has not been maximized and hence was variable..

Our finding that insulin and insulin propeptides in cord blood are higher in females than males is consistent with an intrinsic difference between the sexes which is unlikely to be determined by environmental factors. Other research has suggested that in childhood, females are more insulin resistant than males with higher insulin and proinsulin concentrations and more adipose tissue from the age of 5 years and older and these differences could not be explained by other known determinants of insulin concentrations(1, 3, 4, 6, 19). We think that as females are born smaller (lighter and shorter), with greater skinfold thickness, in the presence of higher insulin concentrations than males, but with no corresponding increase in cord glucose concentrations, this demonstrates they are intrinsically more insulin resistant. This is a similar situation to the difference seen between Indian and UK babies (10) where Indians babies are smaller, with more adipose tissue and with higher insulin concentrations, which is thought to reflect insulin resistance as the increase in cord insulin is not associated with augmented growth. Research has also shown that babies born to the most insulin resistant fathers have higher cord insulin concentrations compared with babies born to the least insulin resistant fathers when matched on birthweight, further supporting the idea of increased cord insulin without increased growth represents an insulin resistant phenotype(20).

The higher proinsulin:insulin ratio and % proinsulins in girls in our study could theoretically be due to either decreased clearance or differences in conversion of proinsulin to insulin (19). Higher proinsulin(s) have been found in neonates previously (11, 12) and this may be part of

the neonatal transition reflecting immaturity of the fetal beta-cell and/or beta cell secretory granule.

Insulin regulates fetal growth, of both skeletal size and soft tissue (21), and as expected, in our data, where sample validity has been established, the insulin concentrations were significantly correlated with all measures of birth size, including weight, length, head circumference and skinfold thicknesses, and had similar correlation coefficients to the relationships seen with total and intact proinsulin concentrations. We also found maternal glucose to be significantly associated with cord insulin concentrations, reflecting the fact that maternal glycaemia is a major determinant of fetal insulin secretion as demonstrated by the macrosomia associated with diabetic pregnancies (22). The findings of these well-established relationships with insulin and total and intact proinsulins provide validation of our results.

Although not the primary aim of our study, our data suggests that stability is dependent on the correct combination of sample type, storage and assay. The difference in sample stability according to sample type and assay we have shown may be one possibility why other studies have failed to see expected relationships of cord insulin with birth weight (23, 24) and birth length (10, 12, 14), and may be why gender differences in only proinsulin, but not insulin, concentrations have been seen (11-13), in contrast to our study. Further studies are needed to clarify the role of specimen type and storage temperature on the stability of insulin in cord blood in particular.

Even with stable sample collection, however, there are still differences in the

relationships seen with insulin and proinsulin. In particular, there was a large difference in insulin concentrations between Caesarean deliveries and normal vaginal deliveries, a difference which has also been seen in other studies (12, 13, 25). No women in the study were given IV glucose as it is not the standard practice of the maternity unit, and the difference remained when adjusting for birthweight, so these factors do not explain this observation. Acute changes around delivery at Caesarean section may be seen more clearly in insulin due to its shorter half-life than proinsulin. Therefore, in future studies looking at cord insulin; it may be more advisable to measure proinsulin(s) as they show less biological variability and may be more stable dependent on collection conditions.

In conclusion, by using appropriate methods of sample collection to ensure insulin results are stable, we have shown evidence from cord insulin and proinsulin measurement to suggest that females are intrinsically more insulin resistant than males.

Acknowledgements:

This study was funded by South West NHS Research and Development, Exeter NHS Research and Development and the Darlington Trust. ATH is a Wellcome Trust Research Leave fellow. BK holds a NHS Research and Development studentship. Thanks to Maurice Salzmann and Annie Goodship for analysis of the cord glucose concentrations. The support of University Hospital Birmingham Charities is gratefully acknowledged (PMC).

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Table 1

Comparison of cord insulin and insulin propeptides between boys and girls, 161 pairs matched for birth weight (to nearest 100g) and gestation (within 5 days). Data presented as geometric mean (SD range). Significance established using paired t-test.

	Boys Geometric mean (SD Range)	Girls Geometric Mean (SD Range)	p
Insulin (pmol/l)	42.1 (23.3-76.1)	49.7 (27.3-90.7)	0.004
Intact Proinsulin (pmol/l)	8.6 (5.3-13.9)	10.5 (6.4-17.0)	0.001
Total Proinsulin (pmol/l)	25.0 (14.5-43.0)	35.6 (18.4-68.8)	<0.001
Birth Weight (g) Mean +/-SD	3535 +/- 414	3534 +/-417	0.847
Gestation (wks) Mean +/- SD	40.1 +/- 1.1	40.2 +/- 1.1	<0.001

Figure 1

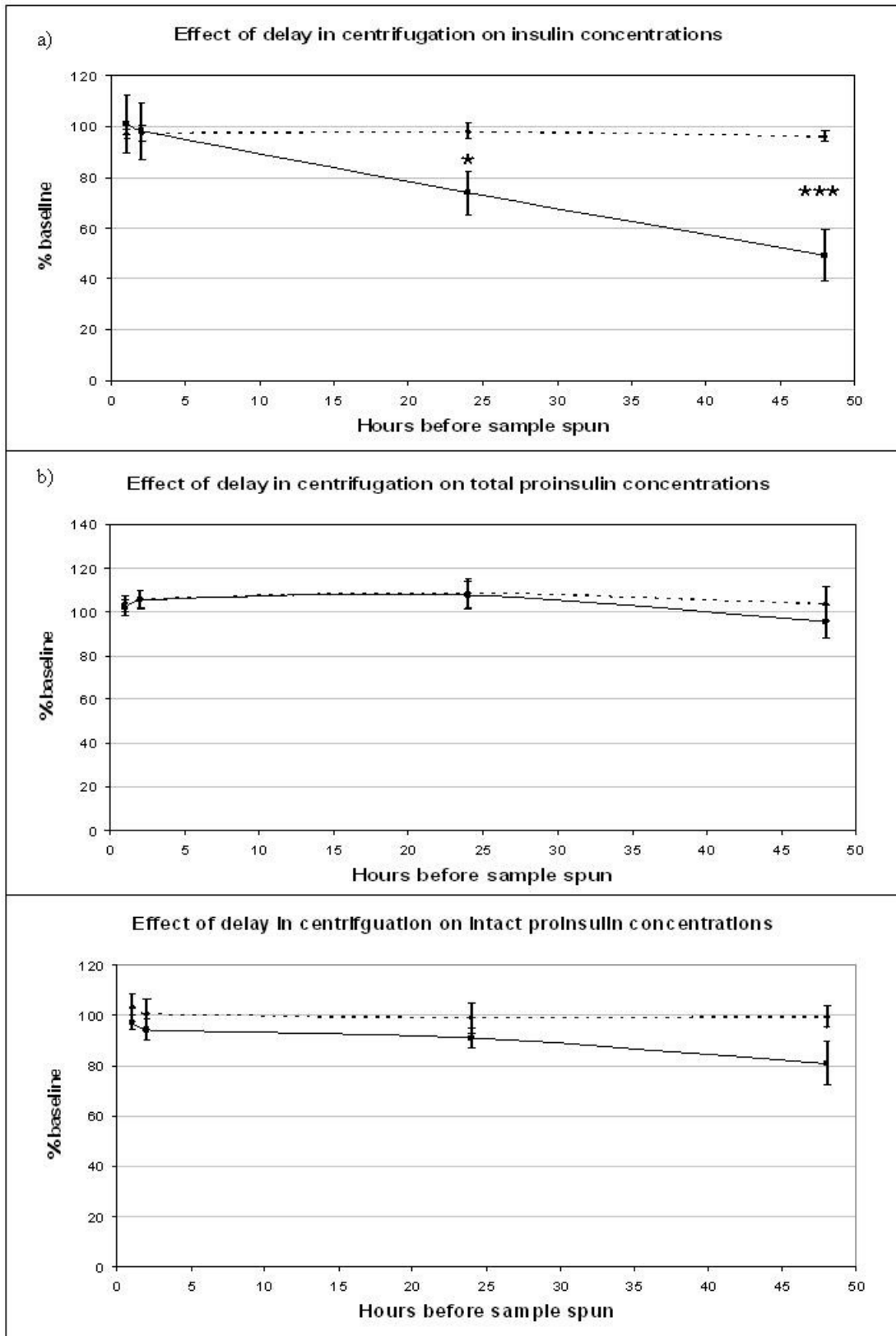


Figure 2

