
Research Article

Heparin Effects on Serum Gonadotropins

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Abbreviations: AMH, anti-Mullerian hormone; BMI, body mass index; CV, coefficient of variation; FFA, free fatty acid; IQR, interquartile range.

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Abstract

Introduction: Studies using lipid infusions to raise fatty acid levels require heparin to release lipoprotein lipase (LPL), thus calling into question the appropriate control infusion for this type of study: saline alone or saline plus heparin. We aimed to evaluate whether the addition of heparin alone, in doses needed to release LPL, would alter circulating free fatty acids (FFAs) and/or affect gonadotropins.

Materials and Methods: This was a secondary analysis using combined data from eumenorrheic normal-weight women subjected to “control” conditions in 1 of 2 separate studies. In 1 study, participants received saline alone (group 1) as a control, and in the other study participants received saline alone and/or saline plus heparin (groups 2-3) as a control. Both studies performed early follicular phase, frequent blood sampling. FSH and LH were compared across groups and in conditions with and without heparin. Linear mixed models were used to analyze the data.

Results: LH did not differ across any of the 3 groups. Estimated means (SE) for FSH differed between groups but this difference was marginal ($P = .05$) after adjusting for anti-Mullerian hormone and unrelated to heparin infusion (group 1: 4.47 IU/L [SE 1.19], group 2: 8.01 IU/L [SE 1.14], group 3: 7.94 IU/L [SE 1.13]).

Conclusions: Heparin does not exert major effects on gonadotropins when infused in quantities sufficient to release LPL. However, because it can release other vascular membrane-bound proteins, heparin should be considered part of the control infusions in lipid infusion studies where increased FFA levels are the goal.

Key Words: FSH, LH, heparin, lipolysis

The term “metabolic syndrome” describes a collection of risk factors that adversely affect many health outcomes, including reproductive fitness [1-4]. Our laboratory and

others have used hyperinsulinemic-euglycemic clamps or insulin infusion in combination with lipid infusion over several hours to recapitulate the insulin- and fatty acid-rich

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intravascular microenvironment of a patient with metabolic syndrome to better understand how obesity impacts reproductive hormone secretion. Studies such as these have been shown to mimic a metabolic syndrome-like state of hyperlipidemia and hyperinsulinemia and cause impaired LH and FSH secretion over several hours of infusion in healthy, normal-weight women [5, 6]. The result of these infusions in normal women mimics the approximately 50% reduction in mean LH and FSH secretion we have observed in obese patients [7]. Our laboratory's preliminary data also indicate that this simulated metabolic syndrome produces a blunted response to GnRH in normal-weight women—again recapitulating findings in women with obesity [6, 8].

Although the protocols used in these studies have offered valuable insight into the pathophysiology of metabolic syndrome and its effects on the hypothalamic-pituitary-ovarian axis, there exists inadequate consensus about the appropriate control exposures to use in these studies. The technical problem of which control to use arises because the elevation specifically of circulating free fatty acid (FFA) levels by continuous infusion of lipid necessitates heparin be given simultaneously to release lipoprotein lipase from the vascular wall. Studies of lipid infusion have varied depending on whether the goal was elevation of FFA levels (heparin included) or triglyceride levels (no heparin), and the control infusions for these studies have also varied in whether the heparin is matched to the lipid infusion being studied. Several studies using lipid and heparin infusions have not included heparin in their control infusions [9-12].

However, heparin is a known disruptor of other protein-membrane interactions and may have independent effects on the experimental outcomes of interest [13]. We performed this study to determine if a saline plus heparin infusion was sufficient to alter FFAs and/or gonadotropin secretion by itself. To our knowledge, a direct comparison of the effects of a saline or a saline plus heparin infusion on FFAs or gonadotropins has not been performed to date. We suspected that minor elevations in serum FFAs might occur with heparin alone. More importantly, prior research in our laboratory have shown that both elevated serum FFAs and insulin are required to lower gonadotropin levels, and that elevated serum FFA alone may cause a nonsignificant increase in FSH levels while causing no change in LH [5]. Although no other studies have directly compared a saline infusion with a saline plus heparin infusion, Mai et al used a saline plus heparin infusion as their control in a study in which they found that infusion of saline plus heparin resulted in a decline of FSH over a 4-hour infusion [14]. They did not observe significant changes in LH. Mai et al compared the saline and heparin infusion with a lipid and heparin infusion and noted that the FSH decline observed in the saline plus heparin participants was less pronounced

than that in the heparin plus lipid infusion. Prior data from our laboratory [5] showed a small increase in FSH after lipid infusion and indicate that heparin may exert nuanced effects beyond general lipolysis. Thus, potentially unsuspected effects of heparin could implicate prior studies that have failed to use heparin in their control infusions and could create false-positive or false-negative findings if the independent effect of heparin is not accounted for. We hypothesized, based on our prior findings, that heparin may have nonspecific effects that could increase FSH levels independent of the metabolic effects of the lipid/insulin infusion.

Materials and Methods

Participants

The current study was performed using data from control visits from 2 previously performed, separate lipid/insulin infusion crossover studies. From 1 study [5], we used control participants who received an infusion of saline only without any heparin (study 1, group 1). Data from a second study [6] was used to supplement the number of participants and allow us to directly compare heparin plus saline infusion with saline infusion alone. This second study included women who received an infusion of saline only without heparin (study 2, group 2) as a control to assess whether heparin had an independent effect on gonadotropin secretion. The women in study 2, group 2, were a subset of the women who have completed a study with saline plus heparin as the control arm (study 2, group 3). The women in study 2, group 3, have had their data previously published as controls to compare their findings with the effect of a lipid plus heparin and insulin infusion on gonadotropins [6]. The parent study to study 2 is registered at clinicaltrials.gov (NCT02653092).

Both study 1 and study 2 used similar inclusion and exclusion criteria. For both studies, recruitment consisted of regularly cycling women (menses every 25-35 days) with a body mass index (BMI) of 18.5 to 24.9 kg/m² who were aged 18 to 39 years. All visits took place in the early-to-mid (study 1) or early (study 2) follicular phase of the menstrual cycle to minimize impacts of the menstrual cycle on glucose metabolism and gonadotropin variability [15]. Both studies recruited women who engaged in vigorous exercise no more than 4 times per week and who reported being otherwise healthy and free from chronic disease. All women meeting these criteria were screened with a history and physical examination performed by a study physician and underwent screening laboratory testing. Both studies excluded women taking medications known to affect insulin metabolism or medications known to alter or interact with reproductive hormones, pregnant women, women

planning to become pregnant, or women without reliable use of contraception, as well as those with an abnormal screening prolactin or TSH. Study 2 was limited to women who had not taken these medications for at least 3 months before their screening visit. In addition, both studies excluded women with abnormal glucose metabolism, defined as an abnormal hemoglobin A1c (>5.7%) in the ongoing study or as an abnormal 75-g oral glucose tolerance test >140 mg/dL or an abnormal fasting glucose >100 mg/dL in the historical study. Both studies were approved by the Colorado Multiple Institutional Review Board. All participants provided informed consent for their participation.

Study Design

This was a comparison study of data taken from control visits of normal-weight, healthy women enrolled in the 2 different studies designed to examine the effect of an infusion of lipid and insulin on various endpoints including serum gonadotropin levels and, for groups 2 and 3, the response to GnRH stimulation.

Study 1 (group 1)

In the group 1 study in which controls were administered saline only [16], participants underwent a maximum of 7 study visits. For the purposes of the current comparison, only data from the visit in which the control saline infusion without lipid or insulin was performed are reported [5]. All visits were restricted to days 5 through 10 of the follicular phase of the menstrual cycle. For 3 days before each of these visits, participants were asked to eat a prescribed and provided diet (50% carbohydrate, 30% fat, 20% protein) and to abstain from moderate to vigorous exercise. Blood samples were obtained immediately before the start of each infusion and every 2 hours thereafter. Participants in this study [16] underwent a saline-control infusion of 0.9% normal saline for 6 hours.

Study 2 (groups 2 and 3)

In the second study [6], women underwent a maximum of 4 study visits, starting with a screening visit that included a blood draw and then followed, in random order, by 2 6-hour infusions visits, each at least 1 month apart: saline plus heparin or lipid and insulin plus heparin. Some participants completed a fourth visit with a 6-hour saline alone infusion. All visits were restricted to days 2 through 6 of the follicular phase of the menstrual cycle. There was no prescribed or provided diet for study 2 participants. Blood samples were obtained immediately before the start of each infusion, and every 10 minutes thereafter. Participants in study 2, group 2 [6], underwent a saline infusion of 0.9% normal saline for 6 hours. Participants in study 2, group 3,

underwent a saline plus heparin-control infusion of 0.9% normal saline with the addition of 24 U/kg/h heparin for 6 hours. This dosing was based on prior studies that used a combination of heparin and lipid infusion to achieve an increase in serum FFA [17]. All infusions started at approximately 8 AM. Although this study included 6-hour infusions, we truncated our analysis for the purposes of this paper to exclude data after T = 240 minutes when participants in study 2 were given a single weight-based (75 ng/kg) dose of GnRH to stimulate a gonadotropin response. Randomization of visit order in both studies was performed using a randomization program.

Hormone and Metabolic Assays

Screening laboratory values, including hemoglobin A1c, a complete blood count, and a complete metabolic panel, were determined by UCHealth Clinical laboratories; serum TSH, serum prolactin, fasting lipids, and partial thromboplastin time were performed at the University of Colorado Clinical Translational Research Center laboratories. Serum FSH and LH were measured using specific, solid-phase immunofluorometric assays (Centaur XP; Siemens) as described previously [18, 19]. Inter-assay and intra-assay coefficients of variation (CVs) were 4.4% and 5.1% for FSH and 4.0% and 2.9% for LH, respectively. Serum nonesterified fatty acids were determined by the University of Colorado Clinical Translational Research Center laboratory using a colorimetric assay (Wako Chemical) using a Beckman Coulter AU480 Chemistry Analyzer with the inter-assay and intra-assay CVs of 1.10% and 5.60%, respectively [20]. Insulin was measured using chemiluminescent immunoassay (Beckman Coulter) with inter-assay and intra-assay of 1.60% and 2.80%, respectively. Anti-Mullerian hormone (AMH) was determined by ELISA (PicoAMH Ansh Labs) from the 0 timepoint before the start of the infusion. The inter-assay and intra-assay CVs were 2.8% and 4.28%, respectively.

Statistical Analysis

Subject demographics were determined from data gathered during screening visits and are shown as a median and interquartile range (IQR [25th and 75th percentiles]) or frequency and percentage. Linear mixed models with random intercepts were performed to assess the relationship between log-transformed LH and FSH outcomes. First models were performed including time (0, 120, and 240 minutes) and group (1: saline only, study 1 [16]; 2: saline only, study 2 [6]; and 3: saline and heparin, study 2 [6]) covariates. Then the models were repeated adjusting for AMH. If there was a difference in the categories of group after adjusting for AMH (*P* values

< .05), then pairwise tests were performed using a Bonferroni corrected significance level of 0.017. Last, for study 2 [6], we looked at descriptive statistics for FFAs and insulin by group and time. We focused on descriptive statistics instead of performing statistical tests because of small numbers.

Results

There was a total of 25 women who underwent 28 studies among the three groups: group 1 contained 9 women from study 1, group 2 contained 4 women, and group 3 contained 15 women as described above. Of the 4 women in group 2, 3 were also in group 3 and underwent both a saline only and a saline plus heparin infusion study on different occasions. Table 1 provides baseline characteristics of the sample by group. Participants were similar in age, BMI, hemoglobin A1c, and distributions of race/ethnicity. AMH was more variable across groups, ranging from a median of 1.35 to 6.43 ng/mL. Based on the day of the menstrual cycle when they were studied, all women were in their follicular phase. None of the women exhibited clinical evidence of polycystic ovarian syndrome. Although we did not conduct ovarian ultrasound examination, all women had normal BMI and hemoglobin A1c, a history of regular menstrual cycles every 25 to 35 days, no hirsutism, and had no evidence of insulin resistance.

There were no differences among the groups for LH in the model adjusted for time or time and AMH ($P = .73$ and $P = .53$; Fig. 1). For FSH, there was a difference in model estimated means when adjusting for time, but that association was attenuated after also adjusting for time and AMH ($P < .01$ to $P = .05$, respectively). In the fully adjusted model, group 1 had the lowest estimated mean of 4.47 (SE 1.19) compared with groups 2 and 3 (8.01 (SE 1.14) and 7.94 (SE

1.13); Fig. 2. Since the P value was just above the significance level, pairwise tests were not performed. Even though the women in the 2 studies differed for FSH, the estimated means were approximately the same for saline only (group 2) and saline and heparin (group 3), both within study 2.

After evaluating descriptive statistics for insulin for study 2 [6], the medians are slightly lower for the saline and heparin group (group 3) compared with the saline only groups (2 at all time points vs 4, 3, and 3; Table 2). We also examined FFAs (Table 3), which were lower at baseline for the saline-only groups (groups 1 and 2) and increased over time. The saline plus heparin median FFA concentrations did not exhibit this increasing trend.

Discussion

Here, we demonstrate that heparin does not exert a statistically significant effect on LH or FSH when used in doses

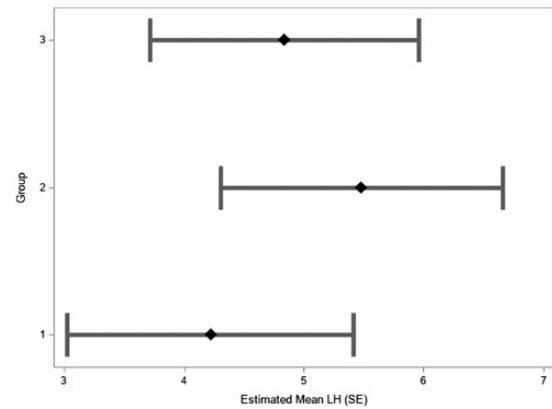


Figure 1. Estimated mean LH (IU/L) and SE for each group adjusted for time and AMH ($P = .53$). Group 1 = study 1; group 2 = study 2, saline only; group 3 = study 2, saline + heparin control.

Table 1. Participant characteristics by study and group

Characteristic	Study 1: group 1 (saline only)		Study 2: group 2 (saline only)		Study 2: group 3 (saline + heparin)	
	N	Median (IQR) or frequency (%)	N	Median (IQR) or frequency (%)	N	Median (IQR) or frequency (%)
Age, y	9	34.00 (28.00, 34.00)	4	33.00 (27.00, 39.00)	15	32.00 (26.00, 36.00)
Caucasian	9		4		15	
No		1 (11.11)		0 (0.00)		3 (20.00)
Yes		8 (88.89)		4 (100.00)		12 (80.00)
BMI, kg/m ²	9	22.60 (21.60, 24.30)	4	22.25 (22.01, 22.78)	15	21.90 (20.15, 22.92)
Fasting glucose, mg/dL	8	81.50 (76.50, 86.50)	4	88.50 (82.00, 93.50)	15	84.50 (80.00, 91.00)
Fasting insulin, uIU/mL	9	7.25 (5.00, 10.00)	4	3.00 (1.50, 5.00)	15	2.00 (2.00, 3.50)
Hemoglobin A1c	8		4		15	
Percentage		5.30 (5.05, 5.60)		4.75 (4.55, 4.95)		5.10 (4.80, 5.40)
mmol/mol		34.4 (31.7, 37.7)		28.4 (26.2, 30.6)		32.2 (29.0, 35.5)
Baseline cycle day	7	4.00 (3.00, 7.00)	3	2.00 (2.00, 6.00)	15	5.00 (3.00, 5.00)
AMH, ng/mL	9	6.43 (4.93, 12.09)	4	1.35 (0.58, 4.72)	14	4.10 (0.98, 5.78)

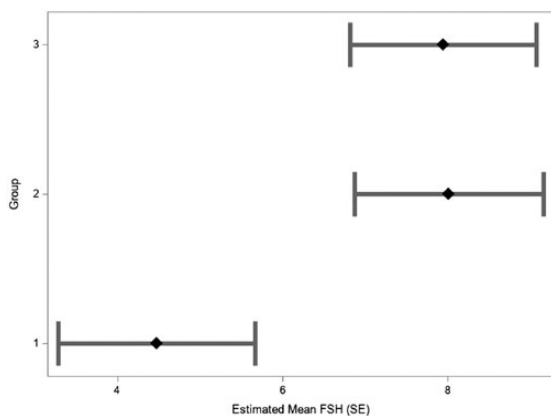


Figure 2. Estimated mean FSH (IU/L) and SE for each group adjusted for time and AMH ($P = .05$). Group 1 = study 1; group 2 = study 2, saline only; group 3 = study 2, saline + heparin control.

Table 2. Median and IQR (25th and 7th percentiles) insulin by group and time for study 2 [6]

Group	Time, min	Insulin median (IQR) uIU/mL
Saline only, n = 3 ^a	0	4 (3, 6)
	120	3 (3, 4)
	240	3 (2, 3)
Saline and heparin, n = 15	0	2 (2, 4)
	120	2 (1, 3)
	240	2 (1, 2)

Abbreviation: IQR, interquartile range.

^aOne person did not have data available.

Table 3. Median and IQR (25th and 7th percentiles) FFAs by group and time for study 2 [6]

Group	Time, min	FFA median (IQR) $\mu\text{mol/L}$
Saline only, n = 3 ^a	0	371 (317, 1047)
	120	585 (360, 730)
	240	618 (478, 789)
Saline and heparin, n = 15	0	607 (471, 849)
	120	771 (708, 953)
	240	711 (603, 824)

Abbreviations: FFA, free fatty acid; IQR, interquartile range.

^aOne person did not have data available.

sufficient to release lipoprotein lipase, and, in the absence of a lipid infusion, heparin alone does not significantly impact serum FFA levels. Although we observed a possible trend toward increased absolute levels of FSH in women who received saline plus heparin, this was more likely from variable ovarian reserve (as measured by AMH), resulting in relatively higher overall FSH levels in the women in group 2—who also did not receive heparin—and group 3

with respect to the women in group 1. Our results differ somewhat from Mai et al, who found that their saline plus heparin control infusions, which used the same concentration of heparin (24 U/kg/h) as our study, caused a small decrease in FSH levels from baseline [14]. The combination of heparin and lipid infusion in the Mai et al study resulted in an even greater decrease in FSH over 4 hours. Their finding that heparin and lipid infusion had more of a negative impact on FSH secretion contrasts with our prior work [5] demonstrating a small increase in FSH in the face of a lipid infusion alone (without heparin) and suggests that there may be an interaction between lipid and heparin that might affect FSH secretion. It is also possible that the overall decline in FSH observed by Mai et al was related to hemodilution. However, given the overall lack of effect in our study and the transient small decrease observed by Mai et al on FSH (and nonsignificant effects on LH), it appears that heparin does not alter gonadotropin secretion in a clinically meaningful manner over short-term infusion. Although heparin is known to release lipoprotein lipase into the circulation, levels of FFA were not significantly increased in response to heparin infusion in the absence of exogenous lipid.

FSH is more likely to be elevated in women with low AMH, and this may underlie the observed variability in FSH levels in our analysis. Indeed, 2 participants enrolled in group 3 were noted to have low AMH (<1 ng/mL); these same women had the highest absolute FSH levels at T = 60, 120, 180, and 240 minutes among women in both studies. These 2 participants likely drove the trend toward elevated absolute FSH levels in the women who received saline plus heparin. On the other hand, 2 participants had relatively elevated AMH levels that could be compatible with a mild polycystic ovary phenotype that includes regularly cycling women [21].

FFAs may exert pro-inflammatory and adverse metabolic effects. FFAs have previously been implicated in increasing circulating androgen precursors [22], as a possible circulating pathogenic factor in preeclamptic pregnancies [23-25], and in inducing insulin resistance [26], all of which suggest that FFAs may affect reproductive fitness. In addition, animal models of diet-induced obesity have shed light on the possibility that elevated FFAs could alter the gonadotropin isoforms present in the pituitary, and therefore change gonadotropin action without changing absolute levels [27]. In this study, heparin infusion alone only resulted in a modest increase in serum FFAs above baseline levels and women receiving saline alone also experienced an increase in FFAs over time because they remained fasting throughout the infusion. Heparin is known to affect aldosterone synthesis [28], and participants may have experienced a decrease in aldosterone production over

the course of the infusion. A reduction in aldosterone, if it occurred, could have attenuated the expected increase in serum FFAs that we observed. However, safety laboratory tests performed at the end of each infusion did not demonstrate any increase in potassium (data not shown) and urine output, which was monitored in participants in study 2, did not differ among any of the experimental conditions or control conditions. The broader context of this work is to better understand how obesity causes reproductive dysfunction in women, and because the condition of obesity is associated with activation of the renin-angiotensin-aldosterone system and concomitant elevation of circulating FFAs [29], interactions between mineralocorticoids, heparin, and FFAs merit further consideration in future studies.

An emerging body of evidence suggests that specific fatty acids, when present in high physiologic serum concentrations, are capable of modulating both hypothalamic GnRH transcription and expression as well as pituitary gonadotropin gene expression and secretion in response to GnRH. In cultured hypothalamic neurons from a female mouse, Tran et al showed that a low concentration of the fatty acid palmitate increased GnRH gene transcription [30]. Similarly, Levi et al demonstrated that a low concentration of palmitate activates *gnrh1* gene expression, but high-dose palmitate suppresses *gnrh1* expression in a mouse hypothalamic cell line [31]. In cultured pituitary cells from a female mouse, Li et al showed that the monounsaturated ω -9 fatty acid oleate, but not the unsaturated fatty acids ω -3 α -linolenic acid or ω -6 linoleic acid, suppresses gonadotropin secretion in response to pulsatile GnRH [32]. These studies indicate that cultured GnRH-producing hypothalamic neurons directly sense circulating FFAs, and that specific fatty acids may be sufficient to modulate *gnrh* gene expression in the hypothalamus in addition to the pituitary response to GnRH that can be modulated by circulating FFAs. Although it is possible that specific fatty acids may have been altered by heparin infusion, these changes were not detectable by our methods and did not induce any acute effects on gonadotropin secretion.

Strengths of our study include enrolling 2 similar cohorts of women, and analyzing, using the same technology, blood samples taken several times (at least every 2 hours) throughout the infusions. However, the 2 studies were conducted several years apart, and although participants were enrolled using very similar criteria, the same participants did not participate in each study, thereby precluding a fully paired analysis. There were also some differences between the studies. The fasting insulin levels of women enrolled in each study differed (7.25 mIU/L in study 1 [group 1, saline only] vs study 2 [group 2, saline only, 3.0 mIU/L; group 3, saline and heparin 2.0 mIU/L]). This is likely because women in study 1, group 1 (saline alone), had been instructed to consume a prescribed diet for 72 hours before the blood sampling session, which may have been higher

in carbohydrates than the study 2 women in the groups 2 and 3 diets, which was not prespecified. All participants were relatively young and healthy and insulin levels were all well within normal parameters despite these differences. Based on prior work involving insulin infusion and its null effect on gonadotropins [5], we would not expect this difference in baseline insulin levels to affect our findings. The women did not differ in other metabolic parameters that we measured. Women from group 1 were asked not to exercise for 3 days before their infusions and this could also be a confounding factor. Although participants' weights in the 2 studies differed (67.1 ± 8.4 kg in the saline study vs 58.1 ± 5.2 kg in the saline plus heparin study, $P = .016$), the difference was likely because average height was greater for the women who enrolled in study 1 because BMI did not differ among any of the groups.

We have shown that, at doses typically used in lipid infusion studies, heparin does not independently significantly impact overall serum FFA levels or modulate gonadotropins. There is still the possibility that elevated FFAs are altering the isoforms but not absolute levels of LH or FSH present in the pituitary, and this mechanism may affect reproductive fitness as well. Although our findings indicate that heparin alone does not significantly increase FFAs, we suggest that adding heparin to saline control infusions is appropriate to control for the effects of heparin on a variety of protein-membrane interactions and the potential downstream effects on the hypothalamic-pituitary-ovarian axis in reproductive studies.

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Data Availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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