ORIGINAL

Hypophosphatemia occurs with insulin administration during refeeding by total parenteral nutrition in rats

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Abstract : Refeeding syndrome (RFS) is characterized by the metabolic and clinical changes that occur following aggressive nutritional supplementation in malnourished patients. Hypophosphatemia is the hallmark of RFS and is key to its prevention and treatment in clinical practice. However, the mechanism of hypophosphatemia during RFS is unclear because of the lack of an animal model. In this study, we developed a rat RFS model as a first step to clarifying the molecular mechanism. After establishing the parenteral route, rats were fasted for 5 days and refeeding was started using total parenteral nutrition. The animals were infused with a high calorie solution with or without insulin administration. Results showed that plasma phosphate levels did not decrease in rats infused with the high calorie solution alone ; in contrast, a 20% reduction compared to baseline was observed in rats administered insulin. In addition, rats infused with the high calorie solution containing added phosphate did not present with hypophosphatemia. Thus, we developed a rat RFS model with hypophosphatemia by tube feeding and insulin administration, and demonstrated the importance of phosphate in preventing refeeding hypophosphatemia. J. Med. Invest. 65 : 50-55, February, 2018

Keywords : *Refeeding syndrome, hypophosphatemia, total parenteral nutrition, animal model, insulin*

INTRODUCTION

Refeeding syndrome (RFS) refers to the metabolic and clinical changes that occur with rapid or excessive administration of feeding, whether orally, enterally or parenterally, following a period of relative or absolute starvation (1). RFS is characterized by fatal complications such as heart failure, arrhythmia, respiratory failure and cardiac arrest (2) ; thus, the prevention of RFS is vital. In general, individuals with marasmus or kwashiorkor are at risk for RFS, particularly in cases of greater than 10% weight loss over a couple of months. Furthermore, patients are at risk after prolonged fasting, massive weight loss in chronic alcoholism, prolonged intravenous fluid repletion and anorexia nervosa (3, 4). Currently, this syndrome is common among elderly people with poor nutritional intake due to alcoholism or dementia in the clinical practice.

Hypophosphatemia is the hallmark of RFS and is key to its prevention and treatment (5). Further, it is reported to be associated with increased mortality in critically ill patients (6, 7). The mechanism of refeeding hypophosphatemia can be described by the following hypothesis. With the reintroduction of carbohydrates to malnutrition patients, anabolism begins immediately and the body shifts to carbohydrate metabolism from fat catabolism. The increased glucose load, with a corresponding increase in the release of insulin, leads to cellular uptake of glucose, potassium (K), magnesium (Mg), and phosphate (Pi). This shift of electrolytes into the cell causes hypokalemia, hypomagnesemia, and hypophosphatemia (8, 9). Patients consistently present with hypophosphatemia, although the other symptoms are not always detected. However, the molecular mechanisms of hypophosphatemia and RFS require elucidation.

RFS is more likely to occur after parenteral or enteral feeding (10). In a study of patients that received total parenteral nutrition (TPN), the incidence of hypophosphatemia ranged from 30-38% when Pi was provided in the solution, to 100% when TPN without Pi was administered (11). The RFS guideline of the National Institute for Health and Clinical Excellence (NICE) in England and Wales recommends that nutritional repletion of energy should be started slowly, and Pi supplementation should be given unless blood levels are high before refeeding (12). Pi supplementation was reported to reduce the incidence of hypophosphatemia in hospitalized patients receiving TPN (13). Thus, Pi has an important role in RFS.

Guidelines on the prevention and treatment of this syndrome are available ; however, cases continue to be seen clinically. One reason why the mechanism of RFS is unknown is the lack of an appropriate animal model. To our knowledge, there are no reports of animal models with hypophosphatemia, making investigation of RFS challenging. Thus, the development of an animal model is vital for the detailed evaluation of RFS as well as clarification of its molecular mechanism. In this study, as a first step toward revealing the mechanism of RFS, we attempted to develop an RFS animal model using the TPN method with a high incidence of hypophosphatemia in rats.

MATERIALS AND METHODS

Animals

Seven-week-old male Sprague-Dawley rats were obtained from Japan SLC (Shizuoka, Japan). Rats were maintained on a 12 h light-12 h dark cycles (9 : 00-21 : 00) with free access to water and standard MF diet (Oriental Yeast, Tokyo, Japan). The experimental procedure was approved by the ethics committee of the University of Hyogo, School of Human Science and Environment.

The parenteral route was established under isoflurane anesthesia

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using an anesthetizer (MT-AT200 ; Muromachi Kikai, Tokyo, Japan). A silicon rubber tube (0.5 mm ID ; As-one, Osaka, Japan) was inserted into the superior vena cava through the external jugular vein and fixed according to the method of Steiger *et al* (14). The tube was delivered to the skull subcutaneously and connected to a swivel via a protective coil attached to the protective harness (Quick connect infusion kit ; Bio Research Center, Nagoya, Japan). The catheter was flushed with 50% heparin (Heparin sodium 5,000 units/5 mL ; Mochida Pharmaceutical, Tokyo, Japan) in saline every second day.

The experimental scheme is shown in Figure 1. Following surgery, the rats were infused with TPN solution (Hicaliq RF ; Terumo, Tokyo, Japan) at a rate of 4 mL/kg/h and given MF diet of approximately 30 kcal during the recovery period for 2 days. Following recovery from surgery, rats were fasted and infused with lactated Ringer's solution (LRS, Solulact ; Terumo) for 5 days. Thereafter, refeeding was started with TPN solution, and blood was collected before and 2, 4, 6, 8, 10 and 12 hours after the start of refeeding. Urine samples were collected for 12 hours (10 : 00-22 : 00) on feeding period after operation, fasting period (fasting day 4) and refeeding period. The RF group (n=5) was infused with Hicaliq RF, which is a glucose-based high calorie and Pi-free solution, during refeeding (Table 1). The RF+I group (n=7) was infused with Hicaliq RF for refeeding and injected with 0.75 U/kg of fast-acting insulin (Humalog® ; Eli Lily, Kobe, Japan) and 5 U/kg of long-acting insulin (Insulin Glargine BSInj ; Eli Lily) before and 2 hours after refeeding, respectively. The RFP+I group (n=4) was injected with insulin in the same way as for the RF+I group, and Hicaliq RF was supplemented with 10 mM Pi as KH_2PO_4 (dibasic potassium phosphate injection 20 mEq kit ; Terumo) for use in refeeding.

Table 1. Composition of TPN infusion

		RF	RFP	LRS
Glucose	(W/V%)	50	50	-
Na^+	(mEq/L)	50	50	131
K^+	(mEq/L)	-	20	4
Ca^{2+}	(mEq/L)	6	6	3
Mg^{2+}	(mEq/L)	6	6	-
Cl^-	(mEq/L)	30	30	110
$Acetate^-$	(mEq/L)	-	-	-
$L\text{-}Lactate^-$	(mEq/L)	30	30	28
$Gluconate^-$	(mEq/L)	6	6	-
Pi	(mmol/L)	-	10	-
Zn	(μmol/L)	20	20	-
Total energy	(kcal/L)	2000	2000	-
pH		4.0-5.0	4.0-5.0	6.0-7.5
Osmotic pressure		11	11	0.9

LRS, lactated Ringer's solution ; Na, sodium ; K, potassium ; Ca, calcium ; Mg, magnesium ; Cl, chloride ; Pi, phosphate ; Zn, zinc.

Biochemical analysis

Plasma levels of Pi, Mg, calcium (Ca) and glucose were determined using the Phosphour C-test, Magnesium B-test, calcium E-test and glucose CII-test Wako kits, respectively (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin concentrations were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Tokyo, Japan). Urinary levels of Pi, Mg and creatinine (Cre) were measured. The measurement was requested to Hyogo Clinical Laboratory Corporation (Himeji, Japan).

Statistical analysis

The significance of differences between groups was assessed using an analysis of variance followed by the Tukey-Kramer multiple comparison test. Changes in body weight and biochemical parameters were evaluated by a paired t test. Data are presented as the mean ± SEM and statistical significance was defined as $p<0.05$.

RESULTS

Establishment of RFS animal model

As a preliminary examination, we investigated the changes in body weight and plasma Pi concentration during fasting for 3, 5 and 7 days. Results showed that the body weight of rats was reduced 17%, 23% and 30%, respectively, while plasma Pi levels were significantly decreased at 5 and 7 days fasting (data not shown). The NICE guideline indicates that individuals without nutritional intake for > 5 days are at risk for RFS. Accordingly, the rats were fasted for 5 days and showed more than a 20% reduction in body weight. As water intake is decreased during food deprivation, the rats were infused with LRS to prevent dehydration during fasting (15). As a next step, we first attempted to refeed using *ad libitum*

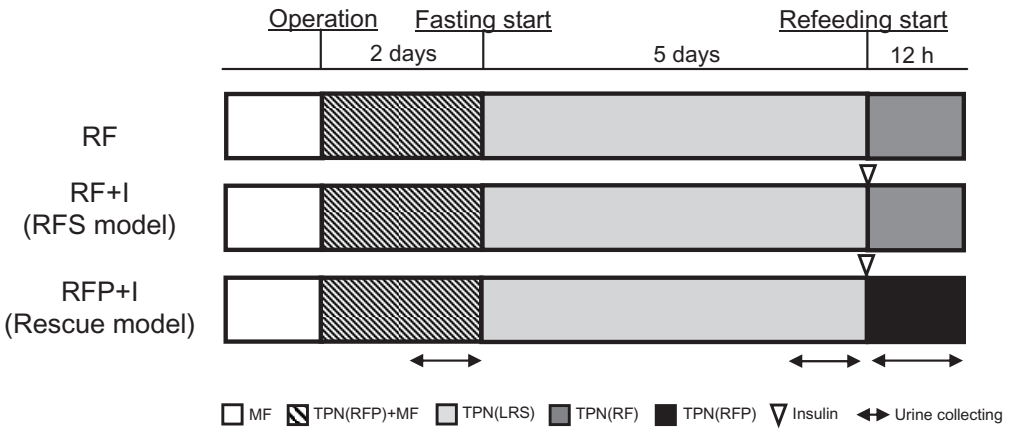

Figure 1. Scheme of refeeding syndrome model

food intake in fasted rats (data not shown). Although the starved rats refed, they did not have adequate food intake commensurate with body weight by fasting for days. We also assessed refeeding using forced oral administration of a high calorie solution ; however, the fasted rats showed intestinal atrophy, decreased intestinal absorption and presented with diarrhea due to the high osmotic pressure of the solution (data not shown). In light of these challenges, we conducted refeeding using the TPN method, allowing for a large infusion of glucose directly into the fasted rats.

In this study, we investigated three protocols of TPN infusion during refeeding (Figure 1). Rats weighting about 230 g were used for the operation, and they received high calorie infusion with TPN and MF diet for the recovery of body weight following surgery. Two days after surgery, the animals were largely recovered, and fasted for 5 days. Next, refeeding was started. Rats were infused with high calorie TPN solution alone in RF group. In RF+I group, rats were infused with TPN solution and injected with insulin, and we defined this group as the RFS model. In RFP+I group, rats were infused TPN solution containing Pi and injected with insulin. We defined this group as the Rescue model, which is capable of preventing RFS. Body weight reduced more than 20% by fasting in all groups, and there were no significant differences in body weight changes between the groups (Table 2).

Table 2. Change of body weight during TPN period

Body Weight (g)	Operation	Fasting start	Refeeding start
RF	231.03 ± 1.38	227.27 ± 2.76	177.99 ± 4.01**
RF+I	234.78 ± 1.43	233.51 ± 2.25	182.98 ± 3.39**
RFP+I	232.47 ± 5.08	235.57 ± 6.52	170.26 ± 2.24**

Values are the mean ± SEM. **$p < 0.01$ vs Fasting start

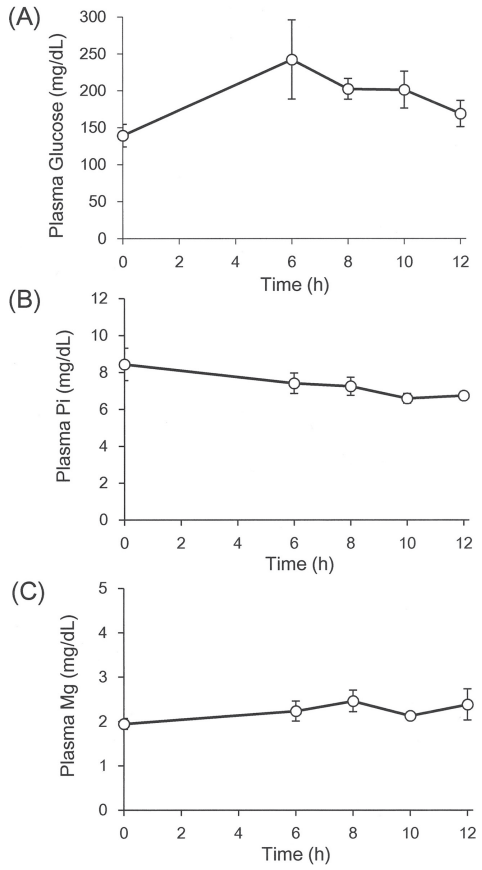

Figure 2. Change in plasma levels during refeeding
RF group (A) plasma glucose, (B) Pi and (C) Mg levels. Values are means ± SEM.

Changes in plasma levels during refeeding

Although we monitored biochemical parameters every 2 h compared to baseline, the parameters became unstable soon after shifting to refeeding (data not shown). In patient reports, refeeding hypophosphatemia was observed at 48 h to 1 week depending on the time required for metabolic changes (16). Hence, we showed plasma levels from 6 to 12 h during refeeding, since they were stable and possibly causative of hypophosphatemia.

Plasma glucose levels were elevated after refeeding in the RF group (Figure 2A). Plasma Pi concentration decreased gradually (Figure 2B) ; however, there were no significant differences compared to the baseline (8.44 ± 0.87 mg/dL at baseline vs 6.74 ± 0.23 mg/dL at 12 h, $p = 0.07$). Plasma Mg levels also didn't change during refeeding with TPN solution alone (Figure 2C). Plasma Ca levels had no change during refeeding (6.74 ± 0.30 mg/dL at baseline to 6.88 ± 0.29 mg/dL at 12 h).

Insulin secretion in refeeding rats

Even though some rats in RF group presented with edema on the face or arm following TPN administration, plasma Pi concentration did not decrease significantly and plasma glucose concentration maintained high levels (Figure 2). This may have been attributable to low insulin secretion during fasting ; thus, we examined plasma insulin levels in refeeding rats similar to RF group (Figure 3). Although plasma glucose levels increased markedly, low levels of plasma insulin were maintained in rats fasted for 5 days and infused TPN solution (plasma insulin concentration increases 1.8 ± 0.4 ng/mL in normal fed rats after infusing glucose solution). Thus, we next administered insulin to starved rats.

Development of hypophosphatemia following insulin injection

To induce RFS, rats were received TPN infusion and insulin injection in RF+I group. Furthermore, to mimic the clinical treatment of RFS, the rats were supplemented with Pi to prevent RFS in RFP+I group. The TPN solution used in the latter group contained 10 mM Pi, which is a common concentration used for normal high calorie infusions that include Pi and K.

Plasma glucose levels were also increased in RF+I group, and these increases were suppressed compared with RF group (Figure 4A). Further, plasma glucose levels also increased in the RFP+I group (Figure 4D), and there were no significant differences between the RFS model and the Rescue model. Interestingly, plasma Pi levels were significantly reduced at 10-12 h in the RF+I group (7.21 ± 0.47 mg/dL at baseline vs 5.82 ± 0.47 mg/dL at 12 h, $p < 0.05$), a 20% reduction compared to the baseline (Figure 4B). These results suggested that the insulin injection caused hypophosphatemia during refeeding. In contrast to the RFS model, plasma Pi levels did not decrease from the baseline in the Rescue model (Figure 4E). Moreover, plasma Pi levels did not increase markedly despite the continued Pi supplementation. This observation supports the case reports that Pi supplementation prevents refeeding hypophosphatemia (11, 13). Although plasma Mg levels did not change significantly in RF+I and RFP+I groups, the change indicated different patterns between the two groups (Figure 4C, F). Plasma Ca levels didn't change during refeeding in these groups

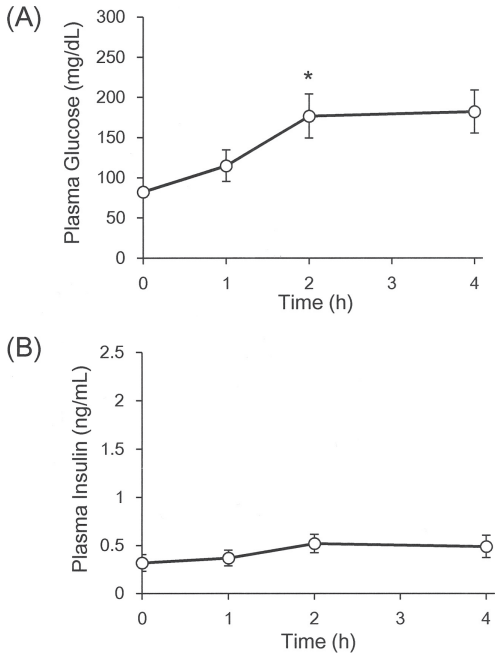

Figure 3. Insulin secretion in refeeding rats
(A) Plasma glucose and (B) plasma insulin levels in refeeding rats similar to RF group. Values are means ± SEM. *$p<0.05$ vs baseline by a paired t test.

even though addition of Pi for the Rescue model (6.28 ± 0.36 mg/dL at baseline to 6.47 ± 0.16 mg/dL at 12 h in RF+I group, and 7.06 ± 0.22 mg/dL at baseline to 6.77 ± 0.32 mg/dL at 12 h in RFP+I group).

Mineral excretions during refeeding

We determined urinary excretions of Pi and Mg during refeeding in order to confirm a decrease of plasma mineral level was not induced by kidney excretion. Urinary Pi levels did not increase during refeeding compared with feeding or fasting period in the RFS model and the Rescue model (Figure 5A). Urinary Mg excretions also did not change significantly in both groups (Figure 5B). This finding showed that refeeding hypophosphatemia was caused by increased cellular Pi uptake *in vivo*.

DISCUSSION

We demonstrated that plasma Pi levels reduced following insulin injection in refeeding rats with TPN and the hypophosphatemia was ameliorated by Pi supplementation. This suggests that our rat model reflects RFS in hospitalized patients. To our knowledge, this is the first study to report the development of an RFS animal model. In addition, we also created a rescue model according to the treatment guidelines.

Several studies have reported that hospitalized subjects with severe malnutrition, i.e., loss of body weight over a short period, low

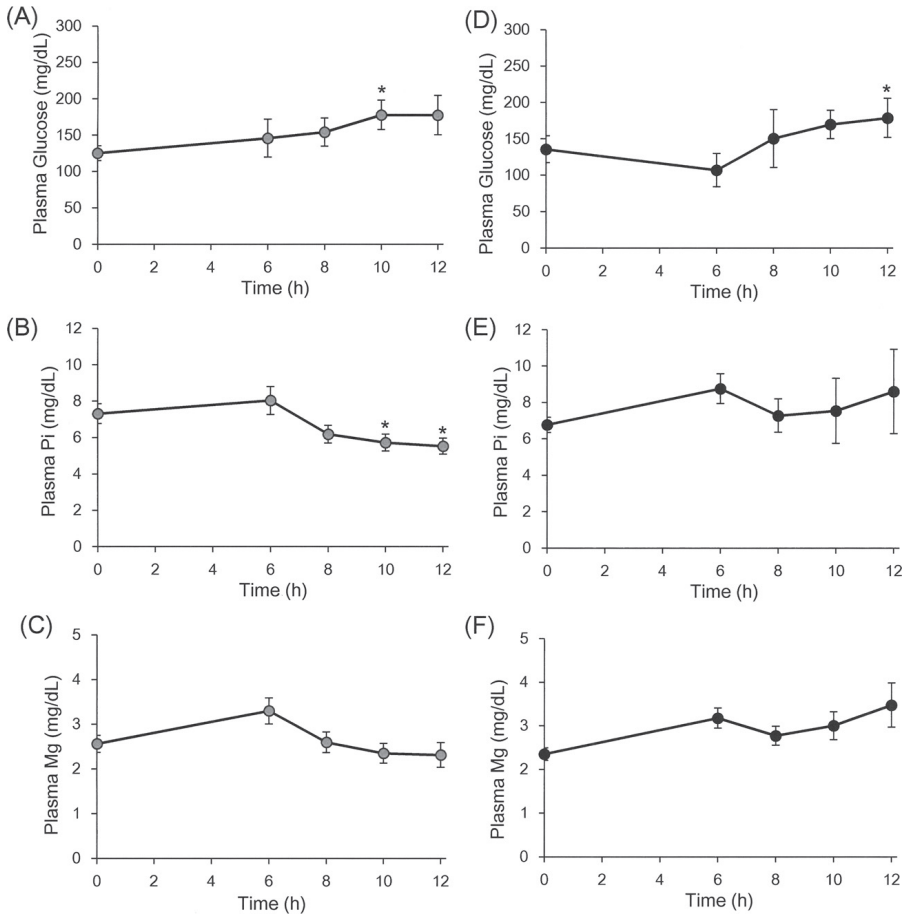

Figure 4. Effect of insulin injection on plasma levels during refeeding
RF+I group (A) plasma glucose, (B) Pi and (C) Mg levels (gray circle). RFP+I group (D) plasma glucose, (E) Pi and (F) Mg levels (black circle). Values are means ± SEM. *$p< 0.05$ vs baseline by a paired t test.

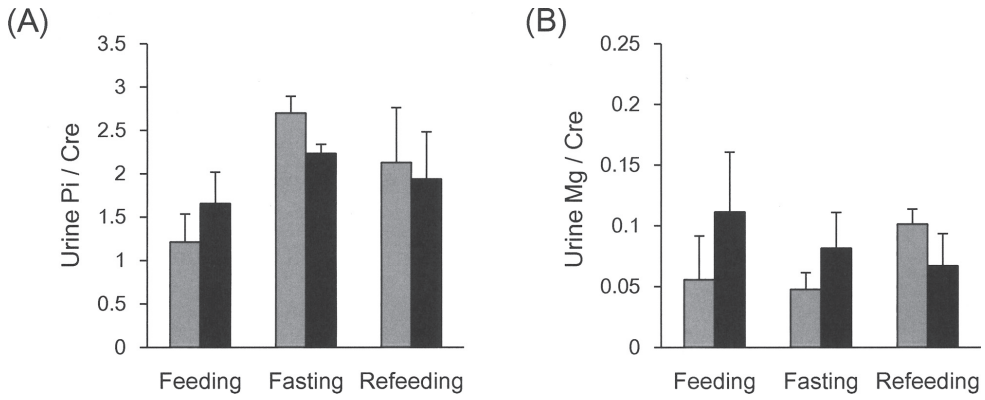

Figure 5. Urinary excretion of Pi and Mg with insulin injection
Urine were collected for 12 hours on feeding, fasting and refeeding period in RF+I group (gray bars) and RFP+I group (black bars). (A) Urinary Pi/ urinary Cre and (B) urinary Mg/ urinary Cre. Values are means ± SEM.

body mass index < 16 kg/m^2 or poor nutritional intake for weeks, developed refeeding hypophosphatemia (17-19). In addition, it was reported that serum Pi levels decreased after about 2 days of refeeding (16). In the present study, rats were fasted for 5 days (resulting in 20% loss of body weight) and developed hypophosphatemia at 12 hours after rapid tube feeding. Furthermore, some RFS model rats showed a reduced breathing rate, edema and a decline in activity after beginning the high calorie infusion, while the Rescue model rats did not show the same degree of those symptoms (data not shown). In general, blood Pi levels in rat is higher than human so that it is difficult to determine how much reduction is hypophosphatemia in animal. However, our RFS model had a reduction of plasma Pi levels and the symptoms as mentioned above. Therefore, we confirmed refeeding hypophosphatemia was caused not by renal excretion but by Pi consumption *in vivo*. These results suggest that this rat model reflects refeeding hypophosphatemia in malnutrition patients receiving aggressive nutrition therapy.

We infused rats with TPN solution at a rate of 4 mL/kg/h in reference to the experimental animal administration guidelines of the European Federation of Pharmaceutical Industries Associations and the European Centre for the Validation of Alternative Methods (20). Other studies in rats used TPN solution containing a fat or protein source, with 270 kcal/kg/day being administered (21). However, we infused 192 kcal/kg/day to rats using a glucose-based high calorie infusion without fat or amino acids to generate RFS. Although this amount of energy might be insufficient for RFS development, it is conceivable that our glucose dosage was sufficient to promote refeeding hypophosphatemia in light of previous reports of the effects of reintroduction of carbohydrates in this syndrome (22). Some reports have demonstrated RFS in animals ; however, symptoms appeared after several days (23, 24). Because of the direct infusion of glucose solution via intravenous administration, we were able to observe hypophosphatemia within one day.

The pathophysiology of RFS is thought to involve the stimulation of insulin release, resulting in anabolic activity and a shift of electrolytes into the cell. We generated hypophosphatemia in TPN-fed rats following insulin injection ; however, plasma Pi levels did not decline significantly during refeeding in the absence of insulin. Furthermore, not only plasma Pi levels but also plasma Mg levels reduction was observed in our RFS model. This result demonstrates that the presence of insulin triggers the development of hypophosphatemia during refeeding. Since insulin activates ATPase (25), increasing insulin concentrations might stimulate Pi consumption for ATP generation, which is essential for energy production. Namazi *et al*. reported that refeeding after starvation decreased serum K and Pi concentrations, increased insulin levels and altered the hepatic histopathology in rats (24). The effect of changes in glucose or lipid metabolism on liver tissues may be associated with insulin-stimulated Pi consumption. In present study, we administered insulin during refeeding because of low insulin secretion in starved rats. However, serum insulin levels in patients identified as at-risk are not measured in the clinical practice. As such, it may be necessary to investigate changes in insulin levels in RFS patients, and the monitoring of insulin levels in at-risk patients may be necessary to prevent refeeding hypophosphatemia in the future. In addition, a previous study revealed that refeeding with glucose induced the gene expression of acute liver inflammation markers and hepatocyte destruction in mice (26). Acute metabolic changes involved in hepatic inflammation are also thought to have a high requirement for minerals during refeeding.

The NICE guidelines outline the prevention and treatment of RFS, specifically focusing on start with hypocaloric nutritional intake and the supplementation of electrolytes in the clinical setting (27). In this context, we were able to prevent refeeding hypophosphatemia by Pi administration, indicating the importance of Pi in energy metabolism during refeeding. In addition, the absence of increased urinary Pi also supported Pi was required *in vivo* on refeeding. Optimization of the supplementation protocol, such as the Pi source, timing of infusion, and effective dose, should be investigated in a future study.

We created an RFS model in order to clarify the mechanism of refeeding hypophosphatemia in rats. This model enables the investigation of the state of RFS in more detail as well as its molecular mechanisms. Following insulin injection associated with hypophosphatemia, plasma Mg levels showed a decrease from baseline (2.56 ± 0.19 mg/dL at baseline vs 2.31 ± 0.28 mg/dL at 12 h, p= 0.072) even though there was no significant difference in the RFS model. This result might to be because rats were infused TPN solution containing Mg^{2+} (6 mEq/dL). On the other hand, plasma Mg levels did not change in the rescue model. Consequently, we should examine the difference in plasma Mg concentrations by with or without Pi during refeeding. Vitamin or mineral deficiencies, such as K or thiamine, have been reported for RFS. Moreover, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were altered in RFS (28). It is therefore necessary to investigate levels of other minerals and hepatic function in our RFS model.

In conclusion, we are the first to report the establishment of an animal model of RFS using tube feeding. This is the first step in determining the mechanism of RFS. We also showed the importance of Pi in preventing refeeding hypophosphatemia. Further study is needed to examine the molecular mechanism of RFS using our rat model.

CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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