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Development of Cookie Test for the Simultaneous Determination of Glucose Intolerance, Hyperinsulinemia, Insulin Resistance and Postprandial Dyslipidemia

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Abstract. A new cookie test was developed for the simultaneous evaluation of multiple risk factors such as glucose intolerance, hyperinsulinemia, insulin resistance and postprandial dyslipidemia. The cookie consisting of 75 g carbohydrate and 25 g fat is ingested and the blood samples are obtained at 0, 1 and 2 hours later. When the two carbohydrate sources, liquid glucose and test cookie, were compared as a glucose load within 3 months, the 2 hr plasma glucose levels were not statistically different, proposing the use of the same criteria at 2 hour glucose level for the diagnosis of diabetes and impaired glucose tolerance (IGT) in subjects without exocrine pancreatic dysfunction. In addition, hyperinsulinemia, insulin resistance (AUC insulin, and/or AUC insulin X AUC glucose), and postprandial hyperlipidemia (ΔTG, Triglyceride; ΔRLP, remnant like particles) have been simultaneously uncovered. Reactive hypoglycemia with adverse epigastric discomfort was observed in 26.3% of the control subjects with liquid glucose, while it was observed in only 1 case (5.3%) without any symptom with cookie tests. In fact, one reactive hypoglycemia out of 5 with liquid glucose turned out to be IGT with cookie test. In 64 subjects with lifestyle-related diseases, cookie test revealed hyperinsulinemia and insulin resistance in 56% respectively, postprandial hyperlipidemia in 39%, diabetes and IGT in 22–23% of each of the subjects and all showed at least one abnormal value. In contrast, in university students with exercise habit, all showed normal results with cookie test. In addition, improved insulin sensitivity over non-exercise group was obverved. In summary, the cookie test provided more informations compared with OGTT using liquid glucose and with fewer side effects. Simultaneous evaluation of glucose intolerance, hyperinsulinemia, insulin resistance, and postprandial hyperlipidemia was also possible.

Key words: Meal test, Postprandial hyperglycemia, Postprandial dyslipidemia, Insulin resistance, Hyperinsulinemia
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SIMULTANEOUS determination of glucose intolerance including diabetes mellitus (DM), hyperinsulinemia, insulin resistance and postprandial dyslipidemia is beneficial for the early detection of metabolic factors involved in the lifestyle-related diseases [1]. Oral glucose tolerance test (OGTT) can reveal glucose intolerance, hyperinsulinemia, and apparent insulin resistance [2], but false reactive hypoglycemia has often been observed with adverse epigastric symptoms and does not reflect the daily blood glucose excursion and insulin responses. The cookie was ingested and the blood glucose, insulin and TG or RLP responses were measured in the newly developed cookie test. With the exception of exocrine pancreatic dysfunction, starch and 15% maltose are thought to be well digested and no significant difference has been reported for the postprandial 2 hr blood glucose levels [3]. Thus after ingestion of the cookie, postprandial hyperglycemia using the same criteria as OGTT, as well as postprandial dyslipidemia can be simultaneously evaluated. Measurement of endogenous insulin gives additional information regarding hyperinsulinemia and insulin resistance [4].

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We have reported a significant correlation between SSPG (steady state plasma glucose) [5], which expresses insulin resistance, and AUC insulin or AUC insulin X AUC glucose under a similar 75 g OGTT [6].

Subjects and Method

Subjects

Healthy control volunteers consists of non-obese healthy students and staff members with ages ranging from 20 to 65 years and gender ratio of F/M, 0.46.

Oral glucose tolerance tests using liquid glucose (Toleran G) or the newly developed cookie tests have been performed within 3 months under conditions of no change of body weight (less than 1 kg) or daily activities relating to various lifestyles in 19 healthy volunteers (Table 1). The cookie test was also performed in 64 subjects with lifestyle-related disorders and in students with or without exercise habit.

Subjects with lifestyle-related diseases were those with obesity (body mass index greater than 25, OB), DM , IGT and the other group. DM group includes known DM and newly diagnosed DM by the present cookie test. IGT are those who have only IGT with cookie test. The other group was comprised of HT [11], hyperlipidemia (HL 5), IFG [1], coronary artery disease CAD [2], and cerebrovascular diseases (CVD, 1) (Table 1). In the other group, OB and DM were excluded, but IGT was included. Those university students with exercise habit performed exercise training more than 3 hours of soccer at least 2 times a week for more than 2 years. Subjects profiles are shown in Table 1. Informed consent was all obtained from all participants after explaining details about the cookie test. The test was conducted under strict guidelines of The Helsinki Declaration on Human Rights revised in 2000.

Table 1. Patient profiles for the control and subjects with lifestyle related diseases and exercise habit (M ± SD)

subject	No.	age	gender (M/F)	BMI
Healthy Control for OGTT & cookie test	19	24.4 ± 11.7	6/13	21.4 ± 1.4
Control for subjects with lifestyle-related diseases*	26	22.3 ± 9.4	16/10	21.3 ± 1.4
Lifestyle-related diseases				
Obesity	24	31.2 ± 15.7	20/4	29.4 ± 4.6
DM	14	62.5 ± 7.6	12/2	25.8 ± 3.2
IGT	6	65.3 ± 15.5	4/2	22.6 ± 4.2
Other	20	65.8 ± 13.0	10/10	21.9 ± 2.7
Subjects with exercise habit	12	19.3 ± 0.7	12/0	21.5 ± 2.7

* includes 19 healthy control.

Cookie test and measurement

The cookie (one pack) consisted of 75 g carbohydrate (85% flour starch, 15% maltose), 25 g butter fat and 7 g protein for a total of 553 kcal. Ten cookies packed in tin foil, which can be stored at room temperature for at least one year were kindly supplied from ABILIT Corp, Osaka Japan. Cookie was ingested with tea or cold water and blood samples were obtained at 0, 1 and 2 hr after ingestion. Subjects were encouraged to ingest cookie within 10–15 min. In rare cases of those who are used to having small breakfast or dislike cookie, they were instructed to eat half of the cookie within 10 min and the remaining half within another 10–20 min. Time count was started at the time when half of the cookie was ingested. Fade away of palatability, degree of stomach fullness, and adverse feeling were monitored by the attending dietician or using questionnaire sheet during the cookie test. These data were used for the diet therapy for overweight or obese subjects. Recognition of fade away of palatability and moderate eating are the new concept of guidelines restricting the excessive eating.

It is reported that various amounts of carbohydrate load (50–100 g, 30 g/square meter of body surface) does not influence the peak blood glucose levels at 1 h, but slightly lower 2 h glucose values were seen with a load of 50 g or 30 g square meter of body surface compared with 75 or 100 g loading [7, 8]. Therefore, the time delay for the ingestion of the latter half of the cookie does not significantly influence the glucose level at peak and 2 hr.

Plasma or serum glucose, total cholesterol (chol), TG, and high density lipoprotein chol (HDL) were measured by enzymatic method using a kit from Kyowa-Medex, Tokyo, and apo B with a latex method from Shima Laboratory Co., Tokyo [9], using Toshiba Autoanalyzer TBA-20FR. Serum insulin was measured by BML Laboratories using radioimmunoassay (Dinabott). RLP was measured by T. Nakano in a collaborative study using a kit from Japan Immunoresearch Laboratories, Takasaki Japan [10]. Polyacryl-

amide gel electrophoresis (PAGE) analyses were performed at 150 volt for 30 min in plasma using Lipophor system (Jokoh Co., Tokyo). Very low density lipoprotein (VLDL) area, midband, small dense low density lipoprotein (LDL) (migration greater than 0.32 cm) and HDL were monitored.

Statistical analyses

Statistical analyses were performed using Microsoft Excel and SPSS. Data are presented as either mean ± SD or SE. Comparison across groups were performed using ANOVA. Comparisons of individual data against control or basal level were performed using regular or paired t-test. Significance by t-test was confirmed by ANOVA. P values below 0.05 were regarded as significant.

Result

Comparison of cookie test with OGTT using liquid glucose in the same subjects

There was no statistical difference between the mean values of blood glucose and insulin responses at 0, 1 and 2 hr following cookie and liquid glucose ingestion (ns by ANOVA), although glucose tended to be lower in the Toleran G group at 2 hr (Fig. 1). The same cut-off point of 11.1 and 7.7 mmol/L is proposed to be used at 2 hr plasma glucose (PG) for the evaluation of diabetes and IGT as WHO criteria for OGTT. With cookie test, 18 were judged as normal, and only 1 with IGT, while with liquid glucose, all were normal below 7.7 mmol/L. However, reactive hypoglycemia tentatively defined as below 4.4 mmol/L at 1 or 2 hr (with a value lower than FPG of at least 0.28 mmol/L) was noted in 5 (26.3%) with liquid glucose, but only 1 (5.3%) in cookie test. Accompanying epigastric symptoms were present only with liquid glucose and none in

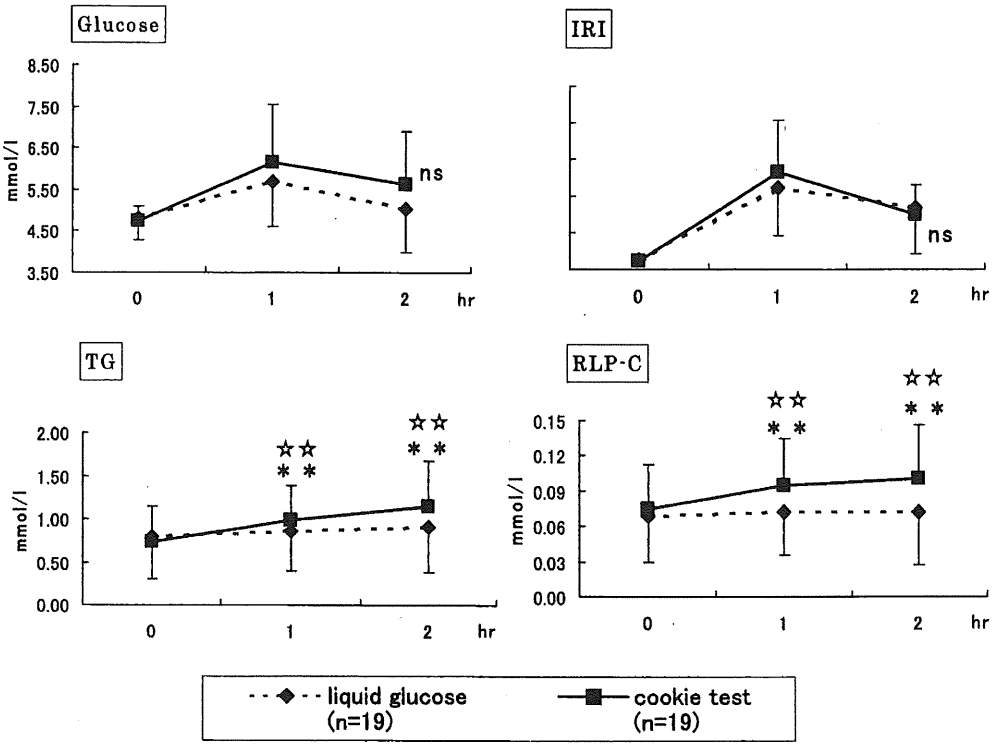

Fig. 1. Comparison of plasma or serum glucose, insulin , TG & RLP-C responses following liquid glucose or cookie ingestion in normal subjects (M ± SD, n = 19)
vs. liquid glucose ☆☆p<0.01 ns vs. liquid glucose by ANOVA
vs. 0 time *p<0.05, **p<0.01

cookie test. The earlier and higher response of insulin was noted in 2 cases with liquid glucose.

With cookie ingestion, significant elevation of serum TG and RLP was noted at 1 and 2 hr (Fig. 1). At 1 or 2 hr those exceeding 0.75 mmol/l for TG and 0.085 mmol/l for RLP-chol above basal was defined as having postprandial dyslipidemia. Insulin resistance was judged from AUC insulin or AUC insulin X AUC glucose, since significant correlation was observed between insulin resistance (SSPG) and the above 2 factors in the regular OGTT in normal or IGT subjects [6]. These cut-off points were calculated from the mean + 2 SD values for the normal control of 26 subjects (Table 1).

Results of cookie test in lifestyle-related diseases

Cookie test was performed in subjects with lifestyle-related diseases, who were classified into 24 obesity, 14 type 2 diabetes, 6 IGT, and 20 others (Table 1). Glucose tolerance was comparable with the previously performed OGTT using liquid glucose in IGT and diabetes group. In diabetes, plasma glucose levels were all greater than 7.7 mmol/L at 0, 1 and 2 hours (Fig. 2A). In IGT and other group, plasma glucose responses show IGT pattern.

Hyperinsulinemia was noted in obesity at 0, 1 and 2 hr, in IGT and in the other group at 2 hour (Fig. 2B). Higher basal level of TG was noted in DM, obesity and the other group (Fig. 2C). Postprandial hypertriglyceridemia was noted in obesity and IGT (Fig. 2C). High basal level of RLP was observed in diabetes, obesity and other group (Fig. 2D). Postprandial high RLP response was noted in obesity. AUC insulin which expresses insulin resistance was significantly higher in obesity, IGT and the other group (Fig. 3). AUC insulin

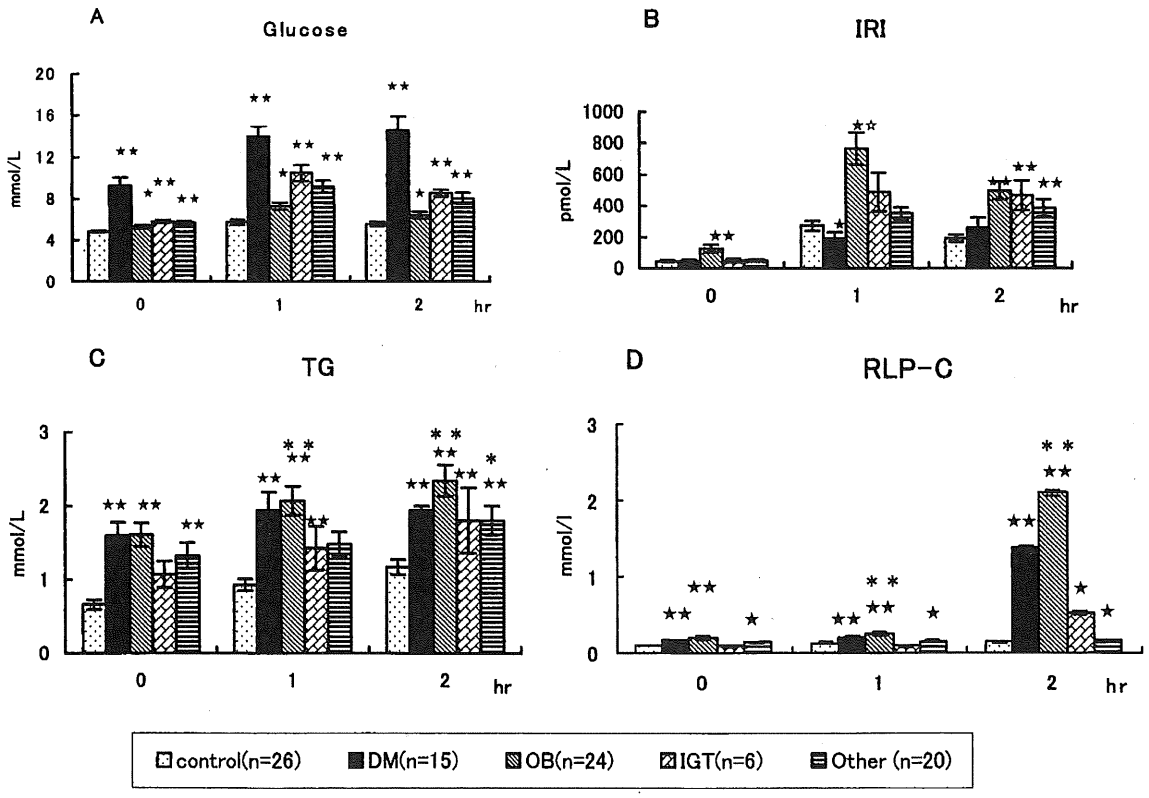

Fig. 2. Plasma or serum glucose, IRI,TG & RLP responses in control & lifestyle-related diseases during cookie test (M ± SE)
vs. control ★p<0.05, ★★p<0.01
ΔTG, ΔRLP vs. control * p<0.05, ** p<0.01

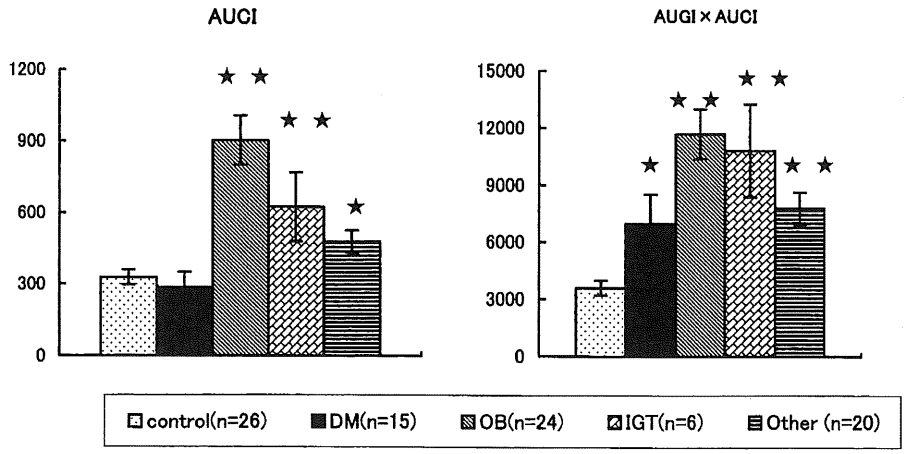

Fig. 3. Insulin resistance index in control & lifestyle-related diseases (M ± SE)
t-test vs. control ★p<0.05, ★★p<0.01

X AUC glucose which is also an index of insulin resistance was higher in all the disease groups (Fig. 3). Basal level of apo B was higher, while basal HDL was lower in DM and obesity and no change was observed during cookie test (data not shown).

Overall incidence of hyperinsulinemia and insulin resistance was each noted in 56%, postprandial hyperlipidemia in 39%, and diabetes and IGT in 22–23% each of the 64 subjects.

Results of cookie test in subjects with exercise habit

In 12 subjects with exercise habit, glycemic responses at 1 and 2 hr were significantly less than those with non-exercise habit (n = 12) following cookie test (data not shown), although both responses were within normal range. IRI responses were also lower at 1 and 2 hr in the exercise group. Subsequently, both AUC insulin and AUC insulin X AUC glucose which reflect insulin resistance are 30–40% less than the non-exercise group. TG responses are also lower at 1 and 2 hr in the subjects with exercise habit. Since those with exercise habit consisted of all male, the control subjects with non-exercise group were 12 male. Their profile are not shown in Table 1.

Criteria of cookie test for defining glucose intolerance, hyperinsulinemia, insulin resistance, and postprandial hyperlipidemia

These criteria were defined as values exceeding M + 2 SD of the control group (n = 26) (Table 2). The same glucose criteria as OGTT were proposed to be used, since under the same equivalence of glucose load (75 g) the same glycemic response was obtained at 2 hr during cookie test. Hyperinsulinemia was defined as those exceeding 86, at 0, 586 at 1 hr and 422 pmol/L at 2 hr. Insulin resistance was diagnozed when AUC insulin and/or AUC insulin X AUC glucose exceeded 790 and 9077, respectively. Those with lower insulin were excluded from the diagnosis of insulin resistance since under the situation of hypoinsulinemia, the term insulin resistance does not apply.

Summary of cookie tests in subjects with lifestyle-related diseases and those with exercise habit

In obesity, hyperinsulinemia and insulin resistance were noted in over 50%, and postprandial hyperlipidemia in 46% (Table 3). In DM, hyperinsulinemia, insulin resistance and postprandial hyperlipidemia were noted in 7–20, 36, and 21% respectively. In IGT and the other group, hyperinsulinemia and insulin resistance were noted in almost half of the subjects. In contrast, in students with exercise habit, none showed abnormal results (Table 3). Increased insulin sensitivity was noted in subjects with exercise habit while by homeostasis model assessment insulin resistance index (HOMA-R) no change was noted.

Discussion

The currently-used liquid glucose (Toleran G,

Table 2. Criteria for the abnormal cookie test

	Basal	1 h	2 h	Δ	comment
Glucose (mmol/l)	6.11, 7.0 ↑		7.77, 11.1 ↑		IFG, IGT, DM
IRI (pmol/l)	86.8 ↑	586.1 ↑	421.8 ↑		Hyperinsulinemia
	28.7 ↓	114.8 ↓	78.6 ↓		Hypoinsulinemia
TG (mmol/l)	1.69 ↑			Δ0.75 ↑	Hyper TG, PPTG
RLP-C (mmol/l)	0.19 ↑			Δ0.09 ↑	Hyper RLP, PPRLP
HDL-C (mmol/l)	1.03 ↓				Low HDL
LDL-C (mmol/l)	3.10 ↑				Hyper LDL
Apo-B (mg/dl)	110 ↑				HB, HBL
Insulin resistance index					
*AUCI (pmol/l·hr)		790 ↑			Insulin resistance
*AUCI × AUCG (mmol/l·pmol/l·hr²)		9077 ↑			Insulin resistance
HOMA-R (mmol/l·pmol/l)/135	2.49 ↑				Insulin resistance

HB (Hyper Apo B), PPTG (Postprandial hyper TG), PPRLP (Postprandial hyper RLP)
HBL (Hyperbetalipoproteinemia): LDL-chol<120, Apo B>110 mg/dl
* Takeuti M, Kanazawa A, Harano Y, 2000, Endocrine Journal 47, 535–542

Table 3. Frequency of abnormal values in subjects with lifestyle-related diseases determined by cookie test

	Ob	DM	IGT	Other	Ex
No. of subjects	24	14	6	20	12
DM[1)]	0	1	0	0	0
DM[2)]	0	5	0	0	0
DM[3)]	0	8	0	0	0
IGT[4)]	2	0	6	7	0
IFG[5)]	1	1	2	5	0
Hyperinsulinemia					
0≥86 pmol/L	18	1	1	3	0
1 h>586	13	1	3	2	0
2 h>422	13	3	3	8	0
Insulin resistance					
AUCI>789 pmol/L·hr	14	1	3	4	0
AUCI*AUCG> 9079 pmol/L·mmol/L·hr²	17	5	4	9	0
HOMA-R≥ 2.49 pmol/L·mmol/L·hr	17	5	2	4	0
Postprandial hyperlipidemia					
ΔTG≥0.75 mmol/L	11	3	2	4	0
ΔRLP≥0.085 mmol/L	10	2	1	0	0
none	0	0	0	0	12

[1)] FPG<7.0 mmol/L, PG (2 h)≥11.1, [2)] FPG≥7.0 PG (2 h)<11.1, [3)] FPG≥7.0, PG (2 h)≥11.1, [4)] FPG<7.0, 7.7≤PG (2 h)<11.1, [5)] 6.05≤FPG<7.0, Other: HT, hyperlipidemia, CAD, CVD

Glucola), which contains 37% glucose and 20% maltose and triose, is not a natural food for the glucose tolerant test [11]. Therefore, the liquid glucose is thought to be rapidly absorbed with early release of insulin. This leads to reactive hypoglycemia at 1–3 hrs with epigastric symptoms such as discomfort, uneasiness, or lethargy. In the present study with liquid glucose among a total of 19 subjects, mild hypoglycemia was observed in 5 (26.3%) with all symptoms in 4 students, and 1 aged healthy volunteer, who showed IGT on cookie test. This incidence of reactive hypoglycemia with liquid glucose was evaluated in the collaborative study with the Central Laboratory for National Cardiovascular Center (NCVC, Suita, Osaka). In a total 2913 cases of OGTT using liquid glucose from Jan 2000 to Aug 2003, reactive hypoglycemia based on the same criteria was noted in 4%, and severe hypoglycemia with plasma glucose less than 2.78 mmol/L was 0.4%. The incidence was much less in the elderly or glucose intolerant subjects who have visited NCVC. Kurachi M reported at the Second Symposium for the Study of Cookie Test, held on May 13, 2005, that using the same criteria as above, the incidence of reactive hypoglycemia with liquid glucose was 9 times higher (111/1371: 8.1%) than with cookie test (4/459, 0.9%) in NTT health check with mean ages, 50–52. These apparent high incidences (4–33%) of artifact of reactive hypoglycemia are clinically important. Hypoglycemia-induced ischemic ECG changes have also been reported [12]. Performance of OGTT in CAD patients using liquid glucose therefore needs caution. Reactive hypoglycemia in cases with damping syndrome can also be more safely detected using regular or half dose cookie test. The therapeutic effectiveness of α glucosidase inhibitor in these subjects has also been demonstrated with the repeated cookie test after drug administration (data not shown).

Regarding carbohydrate source in OGTT WHO criteria defines carbohydrate as equivalent to 75 g glu-

cose. Liquid glucose (Toleran G, Glucola) was developed since the previously-used pure glucose solution gave more side effects. In subjects with exocrine pancreatic dysfunction, we have reported that liquid glucose gives still lower glycemic response over glucose. Maltose is the best natural carbohydrate source for OGTT, since maltose is the digestive end product of carbohydrate in the gut [11]. The cookie is considered as a natural daily food or meal. The overall coincidence rate of cookie test with Toleran G in the diagnosis of glucose tolerance within a year was 66/71 (93%) and 14/20 (70%) in normal and IGT subjects respectively. In 13/13 (100%) known diabetes on diet alone, all were diagnosed as diabetes with cookie test. Kurachi M reported the statistically identical blood glucose values at 0 and 2 hr during cookie test repeated within a year compared with liquid glucose in 158 subjects whose BMI did not change more than 1.

We had developed a SSPG method using constant infusion of external insulin under suppression of endogenous insulin using somatostatin [5]. The present method uses the endogenous insulin and allows rough approximation of insulin resistance, therefore serving as a screening test for the evaluation of insulin resistance. It is better not to use the term insulin resistance in subjects whose insulin response was lower than the lower normal limit (0<30, 1 hr<115, 2hr<80 pmol/L). AUC insulin and AUC insulin X AUC glucose reflect insulin resistance with high specificity greater than 98%, but with low sensitivity of 25%, although this is still better than HOMA-R [13] in which sensitivity is 13% [5]. In subjects with lifestyle-related disorders, insulin resistance was noted in at least nearly half in obese, IGT or the other group, and postprandial hyperlipidemia in 21–46% (Table 3). None showed normal results, while in students with exercise habit, all showed normal values (Table 1, 3). In addition, they exhibited elevated insulin-sensitivity compared with those with non-exercise habit (data not shown).

Postprandial hyperglycemia has been recently reported to be more important than fasting hyperglycemia as a marker of risk for macrovascular complications [14]. The two hr glucose level following cookie test should serve as a good marker for glycemic control. Postprandial hyperlipidemia has been also reported to be associated with insulin resistance syndrome and an effective indicator for developing vascular complications [15]. An abnormal postprandial elevation of TG or RLP has been suggested to lead to endothelial dysfunction [16]. Delayed clearance of postprandial large TG-rich particles is reported in subjects with LPL abnormality [17]. Insufficient activation of LPL due to insulin resistance may cause postprandial dyslipidemia. By PAGE analyses in the present study, major abnormality in the above dyslipidemia was attributable to an increased VLDL and small dense LDL and less frequent appearance of mid-band. Apo B-48 was also noted to be increased following cookie test especially in obese subjects.

Coates *et al.* have developed a 500 kcal mixed meal, which provided more physiological and stable stimuli to the pancreatic beta cells producing glycemic excursion [18]. Fat loading tests (20–45 g fat) have also been reported for the evaluation of postprandial hypertriglyceridemia or RLP [19, 20]. An oral triglyceride tolerance drink has been reported, which contains 50 g fat and 50 g carbohydrate for the efficient monitoring of TG lowering therapies with additional information concerning cardiovascular disease risk [21]. The overall consensus of the meal test is that the test is more physiological and gives more stable results. The cookie test may provide unique opportunities which can evaluate postprandial hyperlipidemia as well as postprandial hyperglycemia.

In this paper we present data showing that a cookie consisting of starch containing 15% maltose and butter is the more practical and better test meal for the evaluation of combined carbohydrate and lipid tolerance test. Although the glycemic response at 2 hr did not change between cookie and liquid glucose, simultaneous respiratory gas analyses revealed elevated fatty acid oxidation and impaired glucose oxidation at 2 hour during cookie test in the obese or diabetic subjects [22]. The cookie is palatable and may be easily ingested among by various ethnic groups. International comparison is therefore possible for the evaluation of glycemic, lipidemic and insulin responses together with insulin sensitivity. In this study, the control subjects were much younger than subjects with lifestyle related diseases. It may be better to perform additional study to re-evaluate the normal criteria according to the age, different ethnic group and disease state including diabetes in a large number of subjects.

In summary, the cookie test provides more data compared with OGTT using liquid glucose and with fewer side effects. Simultaneous evaluation of glucose intolerance, hyperinsulinemia, insulin resistance, and postprandial hyperlipidemia is also possible.

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