Six months of diazoxide treatment at bedtime in newly diagnosed subjects with type 1 diabetes does not influence parameters of β-cell function and autoimmunity, but improves glycemic control.

Running title: Intermittent Diazoxide in type 1 diabetes.

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Objective: Continuous β-cell rest with diazoxide preserves residual endogenous insulin production in type 1 diabetes. However, side-effects have hampered therapeutic usefulness. In a double blind study we tested whether lower, intermittent dosing of diazoxide had beneficial effects on insulin production, metabolic control and autoimmunity markers in the absence of side-effects.

Research Design and Methods: Forty-one newly diagnosed type 1 diabetes patients were randomized to 6 months treatment with placebo or diazoxide, 100 mg at bedtime. HbA1c, C-peptide (fasting and glucagon-stimulated) and FoxP3+ regulatory T-cells (Tregs) were measured. Patients were followed for 6 months after intervention.

Results: Of six drop-outs, three were due to perceived side effects: one subject in the diazoxide group experienced rash, another dizziness, and one in the placebo group sleep disturbance. Adverse effects in others were absent. Diazoxide treatment reduced HbA1c from 8.6% at baseline to 6.0% at 6 and 6.5% at 12 months. Corresponding HbA1c in the placebo arm were 8.3%, 7.3% and 7.5% (p < 0.05 for stronger reduction in the diazoxide group). Fasting and stimulated C-peptide decreased during 12 months similarly in both arms (mean -0.30 and -0.18 nmol/l in the diazoxide, -0.08 and -0.09 nmol/l in the placebo arm). The proportion of Tregs was similar in both arms, and remained stable during intervention, but was significantly lower compared to non-diabetic subjects.

Conclusion: Six months of low-dose diazoxide was without the side-effects, did not measurably affect insulin production, but was associated with improved metabolic control.
Preservation of residual insulin production in type 1 diabetes patients is accompanied by improved glycemic control, reduced microvascular complications, and reduced number of hypoglycaemic events[1,2]. To retain residual insulin secretion is thus highly desirable.

Autoimmune mechanisms are of main importance for β-cell destruction in type 1 diabetes. Accordingly, immunosuppressive treatment retards the destructive process[3-5], and thus has therapeutic potential. But also the degree of metabolic control affects – whether by modulation of autoimmune activity or by other mechanisms - the rate of β-cell deterioration. Thus, in the DCCT study intensive insulin treatment which achieved lower HbA1C than conventional treatment also markedly retarded deterioration in C-peptide levels[2]. This favourable effect could be due to lesser hyperglycemia per se but also to lesser degree of over-stimulation of the β-cells, i.e. “β-cell rest”.

Diazoxide provides “β-cell rest” by reversibly suppressing glucose-induced insulin secretion through opening K-ATP channels in the β-cell [6]. A beneficial effect of 3 mo treatment with diazoxide was documented in 20 newly diagnosed type 1 diabetes subjects. Diazoxide (4-6 kg/kg/24h, i.e. 280-420 mg for a 70 kg person) or placebo was divided into capsules taken three times daily[7]. After the intervention, C-peptide levels were better preserved in diazoxide vs. placebo treated subjects for up to 18 months. Örtqvist et al. obtained similar results with diazoxide 5-7.5 mg/kg/d given to paediatric patients for 3 months [8]. However, disturbing side effects (lanugo hair growth, edema and hypotension) were frequent and have hampered further studies with diazoxide [7,8].

No studies have tested whether a lower dosage of diazoxide would eliminate side effects and still exert a beneficial effect on insulin production and metabolic control in type 1 diabetes. We recently treated type 2 diabetic subjects using a reduced, intermittent dosing of diazoxide, i.e. 100 mg at bedtime[9,10]. Side effects were then absent and insulin production improved provided that patients were simultaneously treated with bedtime insulin[9]. These results encouraged us to perform a similar study in type 1 diabetes.

Beneficial effects of diazoxide in previous type 1 diabetes studies have been proposed to be due to “β-cell rest” and diminishing cellular autoimmune activity [11,12]. However, studies on the effects on T-cell subpopulations are lacking. Among these, much recent evidence points to the importance of regulatory T-cells (Tregs) [13]. Tregs were originally characterized by strong expression of IL-2R, CD25 and recently and more specifically by expression of the transcription factor forkhead box P3 (FoxP3)[14,15]. It was therefore of interest in our trial to look for a relative change in Treg populations.

The aims of this study were thus to investigate in newly diagnosed subjects with type 1 diabetes whether a low-dose and intermittent treatment with diazoxide would:

- be devoid of side effects
- lead to better endogenous insulin secretion, measured by fasting and stimulated C-peptide
- have beneficial effects on metabolic control, measured by HbA1c and home glucose monitoring
- affect autoimmune processes, measured by GAD65 and IA-2 antibody titres and by the FoxP3 marker of regulatory T-cells.

**DESIGN AND METHODS:**

*Design:* Recruitment - Patients were recruited between February 2005 and June 2007 from the university hospitals of St.Olav, Akershus,
Stavanger, Haukeland and North Norway as well as the Levanger Hospital.

Inclusion criteria were: age 18 to 40 years, insulin dependent diabetes with positive test for GADA and/or IA-2A (antibodies against GAD65 and IA-2), fasting C-peptide level ≥ 0.2 nmol/l and diabetes duration ≤ 12 weeks. Exclusion criteria were evidence of drug and alcohol abuse. Further, we did not include women who were pregnant or who did not use contraception.

Patients were given oral and written information before consenting to participate. The study protocol was approved by the Regional Ethics Committee and the Norwegian Drug Agency.

Details of study design (supplemental table 1 in the online appendix available at http://care.diabetesjournals.org).

Before inclusion, all patients underwent a clinical examination and blood sampling for assessment of blood count, lipid profile, renal and liver function. All patients received standard multi-injection regimen consisting of mealtime monomeric-short-acting insulin and long-acting NPH insulin twice daily (except for 2 subjects in the diazoxide group and 3 in the placebo group who received NPH insulin only at night). After obtaining fasting blood glucose consistently between 4-6 mmol/l, and postprandial levels between 5-7 mmol/l, the patients were double-blindly randomised to either diazoxide 100 mg at night, or placebo. Randomization was stratified for age < or ≥ 25 years. The intervention lasted 6 months, and the follow up period another 6 months. The primary end point was β-cell function, assessed by fasting and glucagon-stimulated C-peptide. Secondary end points were glycemic control (HbA1c and blood glucose), insulin dosage, and markers of autoimmunity (GADA, IA-2A and Tregs).

The study visits took place at baseline and once every three months. Visits were focused on glycemic control and occurrence of side-effects. Each visit included a clinical investigation and measurements of fasting blood glucose, blood pressure and body weight. Patients were also examined for presence of oedema and asked about the occurrence of hypoglycaemia and adverse events. Hypoglycemic episodes were registered as minor or major events depending on coping or not. We registered current insulin dosage (fast and long-acting) at each visit.

C-peptide-glucagon stimulation tests were performed in duplicate at baseline and every 3 months thereafter. In total 10 tests were performed in each participant. Blood was sampled in the overnight fasted state and 6 minutes after the intravenous injection of 1 mg of glucagon.

Patients were to perform 7-point home glucose monitoring (7pHGM) during 3 consecutive days at baseline, during intervention, and between 3 and 6 months after intervention. Blood glucose was measured fasting, 2h after breakfast, before lunch, 2h after lunch, before dinner, 2h after dinner and at bedtime using he patients’ own blood glucose measuring device.

Diazoxide was provided by TEVA Pharmaceuticals Europe B.V., The Netherlands. Manufacture of drug and placebo capsules was performed by the Kragerø Tablet Production Unit AS, Norway.

At baseline and at the end of intervention, we isolated peripheral blood mononuclear cells (PBMC) from 11 subjects in the diazoxide, and 9 subjects in the placebo treated group. Cells were isolated by separation on Lymphoprep (Axis-Shield, Oslo, Norway) and then stored below -140°C. We also isolated PBMC from 20 non-diabetic, age and gender matched, blood donors. After thawing, the cells were processed through a FACSCanato™ flow cytometer (BD Biosciences, San Jose, CA). For details see electronic supplement.

**Assays:** HbA1c was assayed by DCA 2000 (Bayer AS Diagnostics, Oslo, Norway).
Reference levels defining normality were between 3.0 and 6.0%. C-peptide; insulin; glucagon and proinsulin were assayed by RIA (Linco Res. Inc., St.Louis, MO, USA). GADA and IA-2A were determined by ELISA (Medizym®, Medipan Diagnostica GmbH, Selchow, Germany). Titers of GADA > 5 U and of IA-2A >10 U were regarded as positive.

Statistics: The coefficient of variation (CV) for fasting C-peptide is 13% [16]. To detect a 20% difference with 80% certainty one would only need 8 patients in each group. Considering a spontaneous variation in C-peptide decline, we planned to include 50% more patients. Assuming a 10% dropout, 36 patients would then need to be included.

For C-peptide glucagon tests we used the mean values of duplicates for analyses. For 7pHGM we used the median for because of high spread in some of the values,. All other results are given as mean ±SEM. Significance testing was performed using the Mann-Whitney test. Dichotomous variables were analysed using the Pearsons Chi Square test.

To assess insulin sensitivity, we used the HOMA (Homeostatic model assessment)-calculator from Oxford university (http://www.dtu.ox.ac.uk)

RESULTS

Drop-outs: Forty-one patients were randomized to either diazoxide (no. 22) or placebo (no.19). Six patients were excluded during intervention, all of these during the first three months of intervention. Three patients dropped out for personal reasons (one from the diazoxide, two from the placebo group). Three patients were excluded due to assumed side effects;from the diazoxide group one due to a rash and another due to dizziness. In the placebo group one patient experienced sleep disturbances, which disappeared after drug cessation. Compared to completers, the drop-outs were slightly older, more overweight, had higher blood pressure and higher fasting C-peptide at baseline, but comparable glycemic control (table 1). The remaining 35 patients completed the study. A random third was asked to return the unused capsules after intervention. The amount of unused medication agreed with the prescribed doses of the study medication.

Baseline characteristics (Table 1): Age- and gender distribution did not differ between groups. Ketoascidosis at first referral was registered in 3 patients in each group. The diazoxide group had significantly shorter disease duration before inclusion vs. the placebo group. There was a significant decrease in C-peptide levels of -0.17 ± 0.04 (p=0.003) in subjects as a whole from the time of diagnosis to inclusion.

Clinical examination and routine blood testing (see “Methods”) were normal (data not shown). Blood pressure, BMI, glycemic control and fasting C-peptide levels did not differ between the groups. The use of nicotine was equally distributed. In the diazoxide group two patients used oral contraceptives, one a statin, one a proton pump inhibitor and one patient gabapentin. In the placebo group, three patients used oral contraceptives and one was on asthma inhalation therapy containing glucocorticoids.

Body weight: The diazoxide group gained 1.1 ± 0.75 kg in total during the study period. The placebo group gained 1.8 ±1.0 kg during the first 3 months, and had a total weight gain of 2.0 ± 1.5 kg. This was significantly higher than in the diazoxide group(p=0.040).

Insulin dosage: There was no significant difference in insulin dosage (total and long acting) between groups (Supplemental table 2).

Glycemic control: HbA1c improved in the diazoxide treated group compared to the placebo group both during intervention and during follow up (Figure 1). Neither fasting blood glucose at the study visits nor the 7pHGM registration showed significant
changes during intervention. However, 3 months after intervention, the fasting blood glucose decreased significantly, by -1.4 mmol/l in the diazoxide treated group while increasing by 1.1 mmol/l in the placebo group (p= 0.017 for difference between groups). The same tendency was seen 6 months after intervention. Only 51% of the patients completed the 7pHGM registrations after the intervention. By these data there was a tendency (P=0.158) for a reduction in blood glucose before and after breakfast in the diazoxide treated vs. placebo group.

Hypoglycemic events: The self-reported frequency of hypoglycaemic events before inclusion was comparable between the two groups. During intervention, 5 patients in the diazoxide group reported frequent minor hypoglycaemic events (>10 events during 6 months). Five patients in the placebo group reported frequently minor hypoglycaemia, and two reported one major event each.

**Parameters of β-cell function:** Fasting and glucagon stimulated C-peptide levels decreased similarly in both groups throughout the study. From comparable baseline values, the fasting C-peptide in the diazoxide group decreased by 0.05 (±0.03) nmol/l after intervention and by 0.13 (±0.02) nmol/l after 12 months. The decrement in the placebo group was 0.02±(0.05) and 0.08 (±0.04) respectively (supplemental table 3). Stimulated C-peptide levels showed the same pattern: from a baseline value of 0.53 (±0.07) nmol/l, the decrement was 0.1 (±0.04) and 0.18 (±0.04) in the diazoxide group. In the placebo group, the baseline value was 0.6 (±0.1) nmol/l and decreased by 0.04 (±0.07) and 0.09 (±0.07) respectively.

To adjust for different glucose concentrations, we calculated the ratio of C-peptide to glucose (Figure 2). No significant difference between groups was observed. Also fasting levels of insulin, proinsulin and glucagon did not change during the study (supplemental table 3).

**Insulin sensitivity:** Insulin sensitivity as assessed by HOMA S% was comparable between the groups at baseline. After 3, 9 and 12 months the HOMA S% increased by respectively 16, 14 and 32 % in the diazoxide group. Sensitivity remained stable in the placebo group (- 3, 0 and 4 % for the same time periods, p= 0.020, 0.147 and 0.072 for differences between the groups).

**Measurements of GADA and IA-2A.** All patients were by selection criteria positive for GADA. Twenty-four subjects were additionally positive for IA-2A. Divided by group adherence 74% in the diazoxide and 63% of subjects in the placebo group were positive for both antibodies. Titres of GADA and IA-2A before intervention were similar between groups. Participants taken together had a mean GADA-titer at start of 182 (±15) U and 168 (±15) after 12 months. The decline in GADA was non-significantly smaller in the diazoxide treated group (p=0.133). The mean IA-2A (all participants) was 188 (±30) U at baseline and 168(±31) after 12 months.

Subjects who were positive for both GADA and IA2A had a tendency for a more pronounced decline in fasting C-peptide after 6 (p=0.092)) and 12 months (p= 0.128) compared to subjects who were only GADA positive, with a similar aggravating effect on stimulated C-peptide after 12 months (p=0.083).

**PMBC and Tregs:** The proportion of CD4+ lymphocytes to the total number of lymphocytes did not differ between the diabetes groups and the healthy control group (supplemental figure 1). The proportion of rTregs and aTregs, expressed respectively as the proportion of CD4+/CD45RA+/CD25+/FoxP3+ cells to the total number of CD4+/CD45RA+ cells, and the proportion of CD4+/CD45RA+/CD25+/FoxP3+ to the total number of CD4+/CD45RA- cells, did not differ between the diazoxide and the placebo treated groups (supplemental figures 2 and 3). The results were the same when the groups
were split into C-peptide decline over or below the median (supplemental figure 4). Likewise, when Tregs were compared in high vs. low titers of GADA and IA-2A, or numbers of antibodies (i.e. one or two antibodies) there was no detectable correlation of the Treg ratio to disease activity (data not shown). However, we found evidence for a small overall down-regulation of Tregs in the diabetic group vs. the non-diabetic group. This significant difference was found both for the rTregs and the aTregs (supplemental figures 2-4).

DISCUSSION

This study is the longest intervention trial with diazoxide so far performed in subjects with diabetes. The main findings are that 6 months of low-dose diazoxide treatment were accompanied with few side-effects, did not measurably affect residual insulin production but was associated with improved metabolic control.

The absence (or near-absence) of well-known side effects [7,8] is in line with our previous studies in type 2 diabetic patients [9,10]. This study extends these observations to a younger age-group and a longer treatment period. A low drop-out rate attests to acceptability of treatment. Compliance with the intervention was further corroborated by satisfactory results from capsule counting. One may conclude that further long term studies with low-dosage diazoxide – if they are indeed indicated - is not hampered by concerns about safety or compliance.

In contrast to the previous studies [7,8], we did not observe any post-intervention effect on fasting or glucagon-stimulated levels of C-peptide. The study of Björk et al, which reported beneficial effects of diazoxide treatment is the study most similar to ours; a discussion of differences of potential importance is thus in order. Age, C-peptide levels at baseline and method of testing insulin production were similar. Participants in the Björk study were included within a week after diagnosis, while intervention in our subjects started up to 12 weeks after initiation of insulin treatment (in line with current recommendations [17]). It seems possible that our subjects were in partial remission at baseline whereas the participants in the Björk study had at that time not entered remission. The implications of such a difference are however not clear. A simple explanation for the lack of measurable effects on insulin production might be the lower dosage of diazoxide. We used a dose of diazoxide that was about one third of the dose used in the Björk study. We reasoned that a longer intervention period, i.e. 6 rather than 3 months in the previous studies, could compensate for a lower dose. Such a putative effect may however have been nullified by a blunting of the effects of diazoxide with time. Thus, there was at least a tendency for inhibition of fasting and stimulated C-peptide after 3 months of intervention but no such effect at the end of the 6 month intervention period. The notion of time-dependent blunting of effects is compatible with the Björk study, in which the suppression of C-peptide during intervention was blunted already at the end of the 3 months intervention period. Björk et al assumed blunting to be due to a recovery of the secretory capacity of the β-cell. This explanation cannot be ruled out, but is less likely in the light of the post-intervention data in our study.

HbA1c improved significantly in the diazoxide group compared to placebo. This effect was seen already during intervention but persisted during the follow-up. The cause of improved glycemic control is not clear. One possibility is improved insulin production that was not picked up by the present methods of testing, i.e. fasting and glucagon-stimulated C-peptide. The fact that the beneficial effect lingered post-intervention
would be compatible with a “hidden” post-intervention effect of diazoxide on β-cell function. One cannot rule out that other tests of β-cell function, such as meal-stimulated secretion would have detected a difference. However, we have no evidence to support this notion. An alternative or additional possibility is that diazoxide favourably affects metabolic control by improving insulin sensitivity. Such a notion is compatible with the insulin requirement not being enhanced in the diazoxide treated group despite the expected inhibitory effect of diazoxide on insulin secretion. Similar observations were made in the two previous clinical studies [7,8] and data from animal studies have indicated improved sensitivity[18] Further, when we calculated HOMA S% (rarely done in type 1 diabetes but deemed admissible[19]) there was significant improvement in the diazoxide treated group during the intervention.

The molecular mechanisms for an insulin-sensitizing effect of diazoxide remain to be clarified. Of possible significance is the fact that body weight increased more in the placebo group during the first three months. In animal studies diazoxide decreases appetite[20], and a similar, albeit unproven, effect could be operative in humans[21].

To our knowledge, ours is the first study to prospectively analyze the relative Treg proportion in PBMC during the early phase of type 1 diabetes. The FOXP3 marker was unaltered through the 6 month intervention period both in the diazoxide and the placebo group. On the other hand the Treg ratio differed significantly between age-matched non-diabetic subjects and the type 1 diabetic subjects. These observations are in contrast to a previous report [22] which however did not focus specifically on differences between newly diagnosed type 1 diabetes vs. healthy subjects. In our study, the samples from the healthy controls were assembled in one center (Trondheim), thus avoiding the transportation necessary for many of the diabetes samples. However we did not find any differences between samples from Trondheim and those from other centers (results not shown). Nevertheless, the observed differences between non-diabetic and diabetic subjects need to be confirmed by an independent study, ideally performed in freshly isolated cells.

As to humoral autoimmunity, we as others [7,8] did not detect any significant influence by diazoxide on GADA-levels.

Are further studies with diazoxide in type 1 diabetes warranted? At first glance the lack of effects on endogenous insulin production would speak against such endeavours. However, since diazoxide at higher dosage does exert beneficial effects, a beneficial effect on insulin production at the present dose in a combination therapy, for instance together with an immunosuppressant, does not seem unreasonable. In any case, the beneficial effects of diazoxide on metabolic control that we observe could be welcome in a combination therapy.

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|                          | Diazoxide          | Placebo           | Drop-outs |
|--------------------------|--------------------|-------------------|-----------|
| Age [years]              | 27.5 ± 1.60        | 27.0 ± 1.76       | 30.8 ± 1.64 |
| Gender [m/f]             | 13/6               | 12/4              | 4/2       |
| BMI [kg/m²]              | 24.8 ± 0.78        | 26.0 ± 1.38       | 27.2 ± 3.53 |
| SBP [mmHg]               | 122 ± 2.7          | 116 ± 3.6         | 126 ± 5.1 |
| DBP [mmHg]               | 75 ± 2.1           | 72 ± 1.9          | 79 ± 3.8  |
| Time of inclusion after diagnosis of diabetes [weeks] | 5.0 ± 1 | 8.0 ± 2 | 7.6 ± 1 |
| Nicotine use [yes/no]    | 6/13               | 6/10              | 2/4       |
| Fasting glucose [mmol/l] | 7.5 ± 0.59         | 7.9 ± 0.46        | 7.8 ± 1.50 |
| HbA1c [%]                | 8.6 ± 0.38         | 8.3 ± 0.56        | 8.1 ± 0.90 |
| C-peptide [nmol/l]       | 0.31 ± 0.03        | 0.34 ± 0.05       | 0.56 ± 0.08 |

**Table 1:** Baseline characteristics (mean ± SEM).  
SBP: systolic blood pressure; DBP: diastolic blood pressure.
**Figure 1:** Changes in HbA1c from baseline, recorded after 3, 6, 9 and 12 months of the study (mean ± SEM). (Baseline mean values for diazoxide: 8.6%; for placebo: 8.3%) Black bars indicate diazoxide-treated, white bars placebo-treated subjects.

**Figure 2:** Figure 2: fasting and stimulated values of C-peptide/glucose ratio at 0, 3, 6, 9 and 12 months. Black circles indicate diazoxide-treated, white circles placebo-treated subjects.

Figure 1
Figure 2

C-peptide/glucose ratio (nmol/mmol)

stimulated values

fasting values

months

0 3 6 9 12