Age-Dependent Development of Metabolic Derangement and Effects of Intervention with Pioglitazone in Zucker Diabetic Fatty (ZDF) Rats

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Running Title: Thiazolidinedione pharmacology in ZDF rats of different age

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Non-Standard Abbreviations:
EDL – Extensor digitorum longus
TZD – thiazolidinedione
ZDF – Zucker diabetic fatty

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ABSTRACT

Zucker diabetic fatty (ZDF) rats are a standard animal model for the study of type 2 diabetes and for pharmacological characterization of insulin sensitizing drugs. To analyze the age-dependent development of their metabolic derangements and the associated changes in their responses to treatment with the insulin sensitizer pioglitazone, groups of 7, 10.5, or 15.5 weeks-old ZDF rats were treated orally with vehicle or pioglitazone (12 mg×kg⁻¹×d⁻¹). Metabolic parameters including circulating concentrations of glucose, insulin, lipids, and adiponectin as well as body weight, tissue glycogen content and the activity of p70S6 kinase in skeletal muscle were determined. Blood glucose of ZDF rats rose steeply from 5.9±0.4 to 23.7±0.5 mmol/l between 7 and 13 weeks of age and then reached a new steady state, which was associated with increased tissue glycogen content (μmol/g in 15 weeks-old ZDF rats vs lean littermates: skeletal muscle, 18.0±0.9 vs 10.5±1.4; liver, 181±6 vs 109±14; both p<0.001). Early intervention with pioglitazone at 7 weeks of age fully prevented the development of hyperglycemia (blood glucose, 6.4±0.4 vs 18.7±1.5 mmol/l after 5.5 weeks of treatment), which was accompanied by a 40% (p=0.01) reduction of the activity of p70S6 kinase in skeletal muscle. These beneficial effects of pioglitazone were progressively lost, if treatment was initiated at later stages of disease development. ZDF rats are thus suitable for preclinical characterization of insulin sensitizing thiazolidinediones in many aspects, but several important differences vs human type 2 diabetes exist and are to be considered in the use of this animal model.
INTRODUCTION

Since obese Zucker diabetic fatty (ZDF) rats have firstly been described (Clark et al., 1983), their metabolic deviations are known to depend on sex and diet. Females slowly develop hyperglycemia only if fed a highly diabetogenic diet, but males are much more hyperglycemia-prone and, in combination with a defined diet (Purina LabDiet Formulab 5008), emerged as a standard model for human type 2 diabetes. The diabetes-like syndrome of ZDF rats is caused by a mutation in the gene encoding the leptin receptor, which results in severe dysregulation of appetite and body weight (Phillips et al., 1996). The predominant factor driving metabolic deterioration is severe adiposity-associated insulin resistance, which explains that ZDF rats are particularly favoured for the study of the pharmacology of insulin sensitizing drugs like thiazolidinediones (TZDs). In males on Purina 5008, regular administration of the TZDs rosiglitazone or pioglitazone fully prevents hyperglycemia or restores normoglycemia, if the treatment is initiated at young age of less than approximately 9 weeks. More severe hyperglycemia as prevailing at an age of 10-12 weeks can only be ameliorated by TZDs, and intervention at 21 weeks of age has been reported to lack any glucose lowering effect (Brand et al., 2003; Finegood et al., 2001; Pickavance et al., 2005; Smith et al., 2000; Sturis et al., 1995; Yang et al., 2004).

The present study was to thoroughly investigate and characterize the age-dependent development of the ZDF syndrome as well as the associated changes in responses to treatment with pioglitazone under standard conditions (males on Purina 5008). The spectrum of parameters measured included several readouts of glucose and lipid metabolism, as well as plasma adiponectin and p70S6 kinase activity in skeletal
muscle. Although p70S6 kinase has been associated with the regulation of cellular insulin sensitivity (Krebs et al., 2007; Um et al., 2004), its possible role in TZD-induced insulin sensitisation has not yet been studied. The aim of the study was to provide a detailed understanding of parallels and differences between the etiologies and pathophysiologies of the ZDF syndrome and human type 2 diabetes, which is an essential prerequisite for the appropriate design and interpretation of preclinical studies using ZDF rats.
METHODS

Rats

Genetically obese ZDF rats were purchased from Charles River Laboratories (Sulzfeld, Germany; breeding facilities in Belgium) and housed in groups of 3-4 rats per cage. As standard in the use of this animal model, rats were male and fed with Purina LabDiet Formulab 5008 (Richmond, IN, USA). They were kept at an artificial 12 h-light/12 h-dark cycle at constant room temperature with free access to food and tap water unless stated otherwise.

Treatment

Oral gavage treatment with pioglitazone (12 mg·kg⁻¹·d⁻¹, in 2 ml/kg 5% gum arabic), a dose with distinct glucose lowering activity in 8 weeks-old ZDF rats (Yang et al., 2004), was initiated in three groups of ZDF rats (fa/fa), which were 7, 10.5, and 15.5 weeks old, and was continued for 5.5, 4.5, and 2.5 weeks, respectively (n=6-12 per group). Controls were treated with the vehicle, and an additional group of vehicle-treated lean littermates not affected by the ZDF syndrome (Fa/-) was studied for comparison starting at 10.5 weeks of age.

On the first and third day of treatment, as well as every 7 days thereafter, rats were fasted for 4 h and weighed. Before the rats received their daily dose of pioglitazone (i.e., approximately 24 h after the last dose), capillary blood for the determination of blood glucose was collected without physical restriction by gently pricking the tip of the tail. On the last day, this procedure was not followed by gavage, but rats were deeply anesthetized with sevoflurane (Abbott Laboratories, Queenborough, England, UK) and subjected to cervical dislocation. A large amount of blood was sampled immediately by
heart puncture and specimens of liver, extensor digitorum longus (EDL) muscle and gastrocnemius muscle (red part), as well as the left epididymal fat pad were collected. Plasma and tissue samples were stored at -20° and -80°C, respectively, until further analysed. Food intake per rat was calculated from consumption per cage.

Analytics

Blood glucose, plasma triglycerides, and plasma cholesterol were measured with an enzymatic analyzer (Falcor 350, Menarini, Florence, Italy) according to the manufacturer’s standard procedures. Plasma free fatty acids were determined with a kit from Wako (Neuss, Germany), and radioimmunoassays from Linco (St.Charles, MO, USA) were used to measure rat insulin and adiponectin in plasma.

p70S6 kinase activity in specimens of EDL muscle is given as % of the protein in the active (phosphorylated) state as determined by Western blotting. Procedures for quantification of the phosphorylation status exploited the different electrophoretic mobilities of the active (phosphorylated) and inactive (dephosphorylated) forms of the kinase and have been described in detail (Krebs et al., 2007). The activity of Akt (also referred to as protein kinase B) was determined in EDL muscle by Western blot measurements of total Akt protein and its activated form, phospho-Akt (Ser473) according to a previously described procedure (Anderwald et al., 2007).

For the determination of glycogen content, frozen specimens of liver and gastrocnemius muscle were lysed in 1 mol/l KOH at 70°C. Glycogen in the lysate was degraded to glucose with amyloglucosidase (Roche Diagnostics, Mannheim, Germany), followed by the measurement of glucose with an enzymatic kit (Human, Taunusstein, Germany).
Results are given as mean±SEM. Differences were analyzed by two-tailed paired Student’s t-test (for comparison of results obtained from the same individuals at different age), or by two-tailed unpaired Student’s t-test (for comparison of results obtained from different individuals) with a p<0.05 considered as significant. Intraindividual associations between two variables are indicated by Pearson’s correlation coefficient (r) with a two-tailed p<0.05 considered as significant.
RESULTS

**Longitudinal development of the ZDF syndrome**

Vehicle-treated ZDF rats showed a distinct increase in blood glucose between 7 and 12.5 weeks of age (from 6.2±0.4 to 18.7±1.5 mmol/l; p<0.001; Fig.1). Thereafter, the development of the syndrome became less dynamic and a new metabolic steady state was reached at an age of approximately 13-14 weeks, which was characterized by basal blood glucose levels of ~25 mmol/l and plasma insulin levels of ~22 mU/l, the latter not different from those prevailing in healthy 15 weeks-old littermates (Fig.2). All other measured parameters likewise remained more or less stable between 15 and 18 weeks of age (Figs.2 and 3). One old vehicle-treated rat, which was still normoglycemic at this age, was excluded from the study (Fig.1).

In 12.5 weeks-old vehicle-treated rats (i.e., in the late dynamic phase of syndrome development) higher individual blood glucose concentrations were associated with lower plasma insulin, body weight, epididymal fat pad weight, plasma triglycerides, plasma free fatty acids, and plasma adiponectin (Fig.4). As predictable from these correlations, plasma adiponectin was lower in individuals with lower body weight, fat pad weight and plasma insulin (Fig.4). In line with the observed intraindividual associations, higher glycemia of 15 or 18 weeks-old than 12.5 weeks-old vehicle-treated ZDF rats went along with lower plasma insulin, triglycerides, and free fatty acids (Figs. 1, 2, and 3; p at least 0.05 each). Likewise, mean plasma adiponectin was somewhat lower in the older, more hyperglycemic vehicle groups (12.5 weeks-old, 4.6±0.4 mg/l; 15 weeks-old, 3.6±0.2, p=0.03; 18 weeks-old, 4.1±0.2, ns). Body weight and fat pad weight remained
constant between 12.5 and 18 weeks of age, while plasma cholesterol increased over this age period (Fig.2).

At 15 weeks of age, vehicle-treated normoglycemic littermates (Fa/-) had similar body weight as hyperglycemic ZDF rats (fa/fa) (377±8 vs 373±8 g, ns), but smaller epididymal fat pads (Fig.2). This hints at a higher relative fat mass in animals of the fa/fa genotype, with lower fat free mass due to a catabolic hyperglycemic state. Accordingly, weight gain between 10.5 and 15 weeks was higher in the healthy rats vs hyperglycemic vehicle-treated animals (Fig.1). Compared to age-matched ZDF rats, normoglycemic littermates further had similar plasma insulin, lower plasma lipids (Fig.2), and markedly lower tissue glycogen content (gastrocnemius muscle, 10.5±1.4 vs 18.0±0.9; liver, 109±14 vs 181±6 µmol glucosyl units/g; both p<0.001; Fig.2).

Effects of pioglitazone treatment

When treatment with pioglitazone was initiated at an age of 7 weeks, the development of hyperglycemia as seen in vehicle-treated ZDF rats was completely suppressed over the whole treatment period of 5.5 weeks (Fig.1). The same intervention at an age of 10.5 weeks caused only a transient amelioration of hyperglycemia (22.2±0.6 vs 17.7±1.1 mmol/l after 10 days of treatment; p=0.004), but the glucose lowering effect of pioglitazone faded progressively thereafter. After 4.5 weeks of treatment, mean blood glucose was similar in rats treated with pioglitazone or vehicle (25.2±0.9 vs 23.5±0.7 mmol/l; ns; Fig.1). When started at an age of 15.5 weeks, treatment with pioglitazone remained without any effect on blood glucose (Fig.1).

At the end of the treatment period, distinctly lower blood glucose seen in pioglitazone vs vehicle-treated rats belonging to the youngest group were accompanied
by a very marked increase in plasma adiponectin (17.8±0.4 vs 4.6±0.4 mg/l; p<10⁻¹⁰; Fig.3). Pioglitazone treatment also resulted in markedly lower plasma triglycerides (-82%) and free fatty acids (-69%; p<0.001 each; Figs. 2 and 3), as well as in elevated weight gain and epididymal fat pad weight (p<0.001 each; Figs. 2 and 3). The activity of p70S6 kinase in EDL muscle was reduced (19.0±3.0% vs 31.7±2.7% in phosphorylated state; p=0.01; Fig.3), but this was obviously not mediated by inhibition of Akt which showed unchanged activity in muscle from pioglitazone-treated rats (97.9±15.3% of control muscles). Furthermore, increased appetite and trends towards lower plasma insulin (p=0.053) and muscle glycogen content (p=0.054) were seen in the pioglitazone-treated animals of young age (Fig.2).

Lack of significant differences between blood glucose concentrations at the end of the treatment periods in the two older groups of ZDF rats was paralleled by absence of significant differences in plasma insulin, triglycerides, free fatty acids, and muscle p70S6 kinase activity (Figs. 2 and 3; except for a modest effect on fatty acids in the oldest group). In spite of loss of any influence on glycemia and these other parameters, pioglitazone maintained its effects on muscle glycogen content, weight gain, fat pad weight and appetite in older rats (Figs.1 and 2). Likewise, pioglitazone elevated plasma adiponectin in the older rats, but the increase was clearly blunted in comparison to the distinct effect seen in association with pioglitazone-induced glucose lowering in young rats (approximately 1.5-fold vs 4-fold; Fig.3). Plasma cholesterol and liver glycogen content were not affected by pioglitazone treatment in any examined age group.
DISCUSSION

ZDF rats as a model for type 2 diabetes

The observed age-dependent changes in circulating glucose and insulin of male obese ZDF rats fed Purina 5008 confirms previous reports about the longitudinal development of their syndrome (Brand et al., 2003; Finegood et al., 2001; Pickavance et al., 2005; Smith et al., 2000). Since higher blood glucose was age-dependently as well as intraindividually associated with lower plasma insulin, deterioration of glycemia can be attributed to an insufficient capacity to produce insulin, which in turn was associated with lower body weight and adiposity (i.e. a catabolic state). So far, the ZDF syndrome resembles type 2 diabetes, but other results point at several important differences, which are to be considered when using these animals as a model of human disease.

Type 2 diabetes is a progressive disease characterized by gradual glycemic deterioration and loss of β-cell function over time, which can ultimately lead to life threatening insulin deficiency (DeFronzo, 1988; UK Prospective Diabetes Study Group, 1995). At variance to the continuous worsening seen in human type 2 diabetes, ZDF rats develop severe hyperglycemia within a short dynamic phase of disease progression, but at a relatively young age of approximately 13-14 weeks they seem to reach a new metabolic equilibrium characterized by severe, but stable, hyperglycemia, hyperlipidemia, and relative insulin deficiency (Erdely et al., 2004; Finegood et al., 2001; Smith et al., 2000; and this study). At this stage, we found glycogen content of skeletal muscle and liver to be approximately 70% higher in hyperglycemic ZDF rats than in their lean littermates, which clearly contrasts with reduced tissue glycogen stores in diabetic humans (Carey et al., 2003; He and Kelley, 2004; Hwang et al., 1995; Krssak et al., 2005).
Different hyperglycemia-associated glycogen stores hint at a much higher potential of glucose to drive its own disposal in rats than humans. As soon as a certain level of hyperglycemia is reached in ZDF rats, glucose seems to promote its own disposal via a mass effect, which compensates for deficient insulin stimulation and allows for long-lasting glucose homeostasis on a severely hyperglycemic, but stable, level. Such species difference in the potential for insulin-independent cellular glucose uptake and glycogen storage could also explain, why only rat muscle shows glucose-6-phosphate accumulation and stimulation of glycogen storage in response to high ambient fatty acids (Randle PJ, 1998; Roden M, 2004). In this context, it is of note that in the ZDF rats a more deranged metabolic state was age-dependently and intraindividually associated with relatively lower circulating free fatty acids and triglycerides. Hence, the role of high plasma free fatty acids as a driving force in the etiology of insulin resistance and diabetes could be different in rats vs humans (Bays et al., 2004; Smith, 2003).

Effects of intervention with pioglitazone

In agreement with previous reports (Brand et al., 2003; Finegood et al., 2001; Pickavance et al., 2005; Smith et al., 2000; Sturis et al., 1995; Yang et al., 2004), early initiation of pioglitazone treatment fully prevented the deterioration of blood glucose, whereas the same intervention failed to restore normoglycemia and even lacked any glucose lowering effect in older ZDF rats. The most straightforward explanation for fading responsiveness to the insulin sensitizer pioglitazone is loss of sufficient circulating insulin, which for obvious reasons is a prerequisite for glucose lowering via insulin sensitization. Particular efficacy of TZDs at the earlier stages of disease development
has also been observed in humans (The DREAM Trial Investigators, 2006), but clinical exploitation of this potential remains limited by concerns about side effects (Nesto et al., 2003; Nissen and Wolski, 2007). Beyond confirmatory findings about age-dependent responsiveness to TZDs, our study is the first to document that secondary failure, as seen with TZD monotherapy in type 2 diabetes (Kahn et al., 2006), can also occur in ZDF rats. Taken together, our results thus confirm certain similarities between TZD effects on glucose homeostasis in diabetic humans and ZDF rats and suggest that the often claimed superior efficacy of TZDs in rodents vs humans could, at least mainly, reflect initiation of treatment at different stages of disease development rather than a species difference.

**Mechanisms of pioglitazone action**

TZDs are believed to exert their insulin sensitizing action at least mainly via adipogenic action and re-modelling of adipose tissue, which improves insulin sensitivity of skeletal muscle and liver via adipocyte-derived signalling molecules like adiponectin (Smith SA, 2003; Whitehead et al., 2006). Even in old ZDF rats, and hence without concomitant glucose lowering, pioglitazone had adipogenic action (reflected by larger fat pads and accelerated weight gain) and increased plasma adiponectin. This is in agreement with the assumption that such events are upstream of TZD-induced changes in glucose homeostasis. Although TZD treatment elevated plasma adiponectin in all age groups, this increase was by far more pronounced in the youngest group of ZDF rats (approximately 4-fold in the youngest vs only 1.5-fold in the older groups). On the first glimpse, this appears to confirm that a robust increase in plasma adiponectin is a prerequisite of glucose lowering action, but our results actually support another
interpretation. Intraindividual and age-dependent associations of higher blood glucose with lower plasma adiponectin in vehicle-treated rats imply that the degree of glucose deterioration predicts plasma adiponectin independently of TZD treatment. In 12.5-weeks old ZDF rats, the association of low adiponectin with high glycemia even outdid the established association of low adiponectin with high body weight (Arita et al., 1999; Bays et al., 2004). The impact of metabolic deterioration (hyperglycemia) on circulating adiponectin is thus by far superior to established direct influences of body weight and pioglitazone, which suggests that most of the TZD-induced increase in plasma adiponectin seen in ZDF rats and type 2 diabetic humans (Miyazaki et al., 2004; Yang et al., 2004; and this study) could be the consequence rather than cause of metabolic improvement.

The next step in the proposed causal chain of TZD action would be that adiponectin acts on insulin target tissues like muscle and liver. In these tissues, adiponectin is known to immediately reduce the cellular energy charge (Yamauchi et al., 2002), which, in concert with a proposed direct effect of TZDs on energy availability (Brunmair et al., 2004; LeBrasseur et al., 2006; Saha et al., 2004), should stimulate fuel recruitment from glycogen for compensatory ATP synthesis. This could explain, why pioglitazone reduced muscle glycogen content independently of the ambient glucose and insulin concentrations. Reduced glycogen in turn increases insulin-stimulated glucose disposal (Laurent et al., 2000; Nielsen et al., 2001) and, hence, could contribute to the beneficial actions of this drug in ZDF rats.

In addition to causing glycogen depletion, cellular energy shortage is known to inhibit p70S6 kinase (Dennis et al., 2001). Although impaired activity of p70S6 kinase has been associated with insulin sensitization via dephosphorylation of serine residues
on insulin receptor substrate-1 (Dennis et al., 2001; Khamzina et al., 2005; Um et al., 2004), accordant TZD effects have been reported only in studies dealing with possible antitumor activities (Cho et al., 2006; Han and Roman, 2006; He et al., 2006). Our study is the first to document reduced activity of p70S6 kinase in the context of TZD-induced glucose lowering and in skeletal muscle, which is the predominant tissue of insulin-induced glucose disposal. Nevertheless, it should be noted that TZD-induced inhibition of p70S6 kinase was observed only in association with glucose lowering and not in the older groups of ZDF rats, which does not allow to differentiate, whether this inhibition is upstream or downstream of insulin sensitization and glucose lowering in the causal chain of TZD action.

**Final Remarks**

Although our study basically corroborates the suitability of ZDF rats for preclinical studies on TZD pharmacology, it also pinpoints differences between the ZDF syndrome and human type 2 diabetes that are to be considered in this context. Beyond this, our study raises the questions, how far changes in tissue glycogen content and p70S6 kinase activity could contribute to the insulin sensitizing action of TZDs, and to what extent TZD-induced increases in plasma adiponectin are the result rather than the cause of metabolic improvement.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

**Figure 1:** Blood glucose (upper graphs) and body weight gain (lower graphs) in male ZDF rats (fa/fa) treated with vehicle (diamonds) or pioglitazone (12 mg*kg⁻¹*d⁻¹; quadruples) during the indicated period of age. A group of 10.5-15 weeks-old vehicle-treated lean littermates was also studied (Fa/-; triangles). Blood glucose is also indicated of an individual, which did not show hyperglycemia up to an age of 18 weeks and was excluded from the study (broken line with circles). Data are given as mean±SEM; *p<0.05; **p<0.01; ***p<0.001 vs vehicle-treated ZDF rats of same age.

**Figure 2:** Metabolic parameters of 12.5, 15, or 18 weeks-old male ZDF rats (fa/fa) after treatment with vehicle (open bars) or pioglitazone (12 mg*kg⁻¹*d⁻¹; full bars) for the indicated time period. Results from 15 weeks-old vehicle-treated lean littermates are also shown (Fa/-; hatched bars). Data are given as mean±SEM; *p<0.05; **p<0.01; ***p<0.001 vs vehicle-treated ZDF rats of same age; †p<0.05; ††p<0.01; †††p<0.001 vs 12.5-weeks-old ZDF rats under same treatment.
**Figure 3:** A-C: Plasma adiponectin, plasma free fatty acids and p70S6 kinase activity in extensor digitorum longus muscle of 12.5, 15, or 18 weeks-old male ZDF rats (fa/fa) after treatment with vehicle (open bars) or pioglitazone (12 mg*kg⁻¹*d⁻¹; full bars) for the indicated time period. Data are given as mean±SEM; *p<0.05; **p<0.01; ***p<0.001 vs vehicle-treated ZDF rats of same age; †p<0.05; ††p<0.01; †††p<0.001 vs 12.5-weeks-old ZDF rats under same treatment. D: Representative autoradiograms showing p70S6 kinase in extensor digitorum longus muscle (upper band = kinase in phosphorylated/active state; V, P: muscle from rats treated with vehicle and pioglitazone, respectively).

**Figure 4:** Intraindividual associations of blood glucose or plasma adiponectin with various other parameters in 12.5 weeks-old vehicle-treated ZDF rats. Pearson´s correlation coefficient (r) and two-tailed probability (p) are given in the graphs.
Figure 1

Graphs showing the changes in blood glucose and body weight gain over different time periods.

7-12.5 Weeks Old

10.5-15 Weeks Old

15.5-18 Weeks Old

Blood Glucose (mmol/l)

Body Weight Gain (g)

Days of Treatment
Figure 2
Figure 3

(A) Plasma Adiponectin (mg/l) levels in different age groups treated for different weeks.

(B) Plasma Free Fatty Acids (μmol/l) levels in different age groups treated for different weeks.

(C) p70S6 Kinase Activity (% phosphorylated) levels in different age groups treated for different weeks.

(D) Western blot analysis of different age groups.

12.5 weeks-old
15 weeks-old
18 weeks-old
**Figure 4**

The diagram illustrates the relationships between glucose levels and various parameters such as insulin, body weight, fat pad weight, triglycerides, free fatty acids, adiponectin, and body weight, fat pad weight, and insulin levels. The correlations are represented by linear regression lines with correlation coefficients (r) and significance levels (p) indicated for each relationship.

- **Glucose vs. Insulin (mU/l):** r = -0.98, p < 0.001
- **Glucose vs. Body Weight (g):** r = -0.88, p < 0.001
- **Glucose vs. Fat Pad Weight (g):** r = -0.90, p < 0.001
- **Glucose vs. Triglycerides (mg/dl):** r = -0.86, p < 0.01
- **Glucose vs. Free Fatty Acids (μmol/l):** r = -0.69, p < 0.05
- **Glucose vs. Adiponectin (mg/l):** r = -0.81, p < 0.01
- **Adiponectin vs. Body Weight (g):** r = 0.74, p < 0.05
- **Adiponectin vs. Fat Pad Weight (g):** r = 0.69, p < 0.05
- **Adiponectin vs. Insulin (mU/l):** r = 0.82, p < 0.01