

CEREBROVASCULAR SAFETY OF SULFONYLUREAS: THE ROLE OF K_{ATP} CHANNELS IN NEUROPROTECTION AND STROKE RISK IN SULFONYLUREA TREATMENT OF TYPE 2 DIABETES

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Introduction

Sulfonylureas are the oldest class of anti-diabetic drugs and are currently the most widely used due to effectiveness and low cost. Sulfonylureas target insulin secretion by blocking ATP-sensitive potassium (K_{ATP}) channels, depolarizing pancreatic β -cells and triggering the release of insulin. Sulfonylureas (SU) binds to the SUR subunit of the Kir/SUR hetero-octamer reducing mgADP's binding and efficacy of ADP induced opening, ultimately results in closure of K_{ATP} whilst masking ATP inhibition of the channel ². This becomes problematic, as K_{ATP} channel activity have demonstrated neuroprotection against ischemic brain injury ^{3,4}.

Diabetes mellitus type 2 (T2DM) is a metabolic disease where hyperglycemia persists and common comorbidities including hypertension, high blood cholesterol, atherosclerosis and obesity contribute to increasing risk for stroke⁵. Not surprisingly, T2DM is a major independent risk factor for stroke as consistently observed in multiple racial backgrounds ⁶⁻⁸. Diabetes and stroke are leading causes of death and disability worldwide therefore risk modification and preventing more severe strokes in diabetic patients is crucial. Adequate glycemic control can lower risk for stroke and improve stroke outcomes but because SUs prevent K_{ATP} channel activation, which is neuroprotective, their use may increase incidence of stroke in diabetic patients. Many studies are focused on the cardiovascular safety of SUs however very few address stroke risk associated with SU diabetes treatment. Thus, we investigated in role of K_{ATP} channels in neuroprotection against ischemic insult and the stroke risk associated with treatment of diabetes, particularly with K_{ATP} blocker SU. We present our in vivo findings from animal models as well as a systematic meta-analysis of randomized clinical trials.

Materials and Methods

STZ administration and MCAO Animal Model. To induce hyperglycemic diabetic model, adult male C57BL/6J mice were fasted for 4 hours and injected with 50mg/kg streptozotocin (STZ) in sodium-citrate solution or vehicle intraperitoneally once daily for 5 days. For the transient middle cerebral artery occlusion (tMCAO) model, 20mm-long monofilament suture with silicon-coated tip was inserted through the right common carotid artery, into the internal carotid artery to block the origin of middle cerebral artery (MCA)³. In tMCAO, suture was removed after 90 min. tMCAO was chosen for STZ-treated mice because permanent MCAO (pMCAO) may affect survival. For pMCAO, suture was left for 24h until next procedure. K_{ATP} channel blocker, tolbutamide (100 mg/kg, i.p. Sigma- Aldrich Canada), K_{ATP} channel opener, diazoxide (20 mg/kg, i.p. Sigma- Aldrich Canada), or vehicle (DMSO) was administrated 20 min before pMCAO.

Measurement of Infarct Area and Assessment of Neurobehaviour. 24h after MCAO procedure, brains were coronally sectioned into 1mm slices and incubated in 2% Triphenyltetrazolium chloride (TTC) at 37°C for 30 minutes. Infarction volumes were calculated by summing areas in slices and multiplied by thickness to obtain volume. Areas were corrected for edema. Corrected infarct volume (CIV) (%) = [contralateral hemisphere volume – (ipsilateral hemisphere volume – infarct volume)]/contralateral hemisphere volume X100 ⁴. Neurological deficits were evaluated 24h after MCAO using 6-point scale. Scale of scores: 0–no neurological deficit, 1–retracts left forepaw when

lifted by the tail, 2—circles to the left, 3—falls while walking, 4—does not walk spontaneously, 5—dead

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Primary Cortical Culture and Oxygen and Glucose Deprivation (OGD). E16 CD1 mice brains were dissected and plated on poly-d-lysine coated plates using Neurobasal culture medium at 1×10^5 per well and maintained at 37°C in humidified 5% CO_2 incubator. Cortical cultures were incubated with tolbutamide or diazoxide in oxygen-glucose deprived solution for 30 min and incubated in anaerobic chamber (5% CO_2 and 95% N_2 v/v) at 37°C for 90 minutes¹⁰. Cells were allowed to recover for 24 hours in normoxic condition.

Immunocytochemistry and Confocal Imaging. Cell death was determined by quantitative measurement of PI fluorescence with excitation wavelength 488nm and recorded emission wavelength of 630nm¹⁰. Cells were incubated with PI (5 $\mu\text{g}/\text{ml}$) for 20 min. Cells were stained with PI for 20 min and fixed with 4% paraformaldehyde for 20 min. Images were taken using a confocal microscope.

Western Blot Protein Analysis. Determine levels of stroke related proteins in brains of STZ-injected and vehicle treated mice by western blot. Antibodies used included: Anti-N-methyl-D-aspartate (NMDA) receptor 2B (NR2B), anti-postsynaptic density protein 95 (PSD95), anti-phosphorylated glycogen synthase kinase 3b (p-GSK3b), anti-total GSK3b (t-GSK3b), and anti-GAPDH.

Data Analysis. Statistical differences were determined using Students *t* test for two-group analysis and one-way ANOVA with Fisher least significant test for multiple groups. Data are presented as mean \pm SEM. Value of $P < 0.05$ was considered statistically significant. All experiments were performed in a blinded manner.

Meta-analysis of Randomized Trials. A meta-analysis was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines¹¹. Using search terms: “sulfonylureas” or “glyburide” or “glibenclamide” or “tolbutamide” or “acetohexamide” or “gliclazide” or “glipizide” or “chlorpropamide” or “glimepiride,” in combination with “stroke” or “cerebral infarction” or “cerebral ischemia” (or “ischaemia”) or “cerebrovascular disease” or “cerebrovascular attack” or “brain ischemia” (or “ischaemia”) on databases: Medline, Web of Science, Library of Congress, Embase, and the Cochrane Library up to 9 Feb 2016.

Results

STZ-Induced Diabetes increases Severity of Stroke

STZ-induced diabetic mice displayed larger areas of brain damage after tMCAO compared to vehicle treated control group. Infarct volume in the STZ group ($50.39 \pm 7.66\%$, $n = 8$) was significantly larger than that of the vehicle-treated group ($32.44 \pm 2.93\%$, $n = 8$; $P = 0.046$) (Fig. 1D). The neurological deficit score in STZ-induced diabetic mice (4.13 ± 0.30 , $n = 8$) was significantly higher than vehicle-treated control group (3.00 ± 0.42 , $n = 8$; $P = 0.047$) (Fig. 1E). These results indicate more severe strokes in diabetic mice compared to vehicle-treated control mice. STZ-induced diabetes was confirmed by lower body weights and higher blood glucose levels shown in Fig. 1A and B).

Diabetes Up-regulated NR2B and PSD95 Protein Levels but Decreased p-GSK-3 β in Diabetic Mice

In STZ-induced diabetic mice model, we compared protein levels of p-GSK3b, NR2B, and PSD95 using Western blot (Fig. 1F). We found that levels of p-GSK-3 β (inactive form of pro-apoptotic protein) was decreased but not t-GSK-3 β in STZ-administered mice (Fig1. G). Additionally, NR2B (NMDAR subunit 2B) and PSD95 were significantly increased (Fig1. H and I). The NR2B subunit is linked to cell death induced by excessive calcium via NMDAR during neuronal injury¹² while PSD95

forms a complex with NR2B that when disrupted lead to decreased neuronal death in stroke¹³. These findings are consistent with the increased severity of stroke in STZ-induced diabetes and hint at the underlying mechanisms.

K_{ATP} Channels Protect Against OGD-Induced Primary Cortical Neuronal Injury *In Vitro*

Tolbutamide treated cultures showed significantly higher OGD-induced neuronal cell death (61.93±3.15%, n = 5) compared with vehicle-treated cells (39.78±1.74%, n = 5; P < 0.001) (Fig. 2A). Conversely, neurons treated diazoxide showed less cell death (21.78 ± 3.38%, n = 5) than vehicle-treated neurons (38.07±4.68%, n = 5; P = 0.004) (Fig. 2C). PI fluorescence images also indicated that PI-positive cell counts increased significantly in the tolbutamide group (313.8±34.77, n = 5; P < 0.05) (Fig. 2B and E), but decreased significantly in the diazoxide group (79.92±12.71, n = 5; P < 0.05) (Fig. 2D and G) compared with vehicle-control groups (175.52±24.42 and 162.44±14.67, respectively, n = 5 per group). These results indicate that activation of the K_{ATP} channel protects against OGD- induced neuronal cell injury *in vitro*.

K_{ATP} Channels Provide Neuroprotection Against Stroke *In Vivo*

The infarct volume after pMCAO was increased significantly in the tolbutamide group (52.99 ± 4.46%, n = 5; P < 0.05) (Fig. 2I and J) but was decreased significantly in the diazoxide group (25.01±2.39%, n = 5; P , 0.05) (Fig. 2K and L), compared with the vehicle-control groups (38.12 ±3.85% and 42.43± 3.78%, respectively, n = 4 per group). The neurological deficit score was higher in the tolbutamide group (n = 5, P = 0.193) and significantly lower in the diazoxide group (n = 5, P = 0.046) compared with the vehicle-control group (Figs. 2F and H). These results indicate that activation of neuronal K_{ATP} channels provides neuroprotection to stroke *in vivo*.

Meta-analysis Showed That Use of Sulfonylureas Poses a Greater Risk of Stroke for Patients With T2DM

Figure 3A shows the selection process and exclusion criteria. 17 studies were selected from the articles retrieved. No major asymmetry appeared in the funnel plot (Fig. 3B). The Cochrane system estimate of quality of trials is shown in Fig. 3C. 41.18% were in the low-risk level, 58.82% unclear level, and no studies were high risk therefore total quality of the included articles was high. Data were combined using random-effects models. Patients who received SU treatment had a higher OR for stroke morbidity of 1.39 (95% CI 1.16– 1.65) than those who received comparator drugs (Fig. 3D). The OR did not differ significantly in subgroup analysis by sex, age, duration of diabetes, BMI, or the HbA1c level (Fig. 3E). In direct comparisons, the increase of risk with SUs reached statistical significance versus dipeptidyl peptidase 4 inhibitors. Meta-analysis showed that T2DM patients treated with SUs have higher ratio of stroke morbidity.

Conclusion

Our data suggests that the use of SUs reduces neuroprotection provided by the K_{ATP} channels in ischemic events and increases the risk of stroke in type 2 diabetic patients as compared to other anti-diabetic drugs. STZ-induced diabetes increased brain damage induced by stroke and STZ-induced diabetes increased expression of NR2B/PSD95 proteins, indicating potential of increase stroke risk and severity with diabetes. K_{ATP} channel blocker, tolbutamide, increased brain and neuronal injury in *in vivo* and *in vitro* models of stroke, while K_{ATP} channel opener, diazoxide, reduced brain and neuronal injury *in vivo* and *in vitro*. This evidence supports the neuroprotective role of K_{ATP} channels and confirms our previous findings^{3,4}. Our meta-analysis of randomized trials show that patients with T2DM receiving either monotherapy or combination therapy containing SUs have a higher relative risk for stroke morbidity than those receiving comparator drugs. Altogether our data conforms to the literature in that diabetic patients have higher risk for and more severe strokes. Additionally, SU management of diabetes increases risk for stroke perhaps through lessening neuroprotective role of K_{ATP} channels. Due to cost and efficacy, SUs continue to play an important role in the management of hyperglycemia, however as the diabetic population grows, mitigating risk for complications is critical. Therefore careful assessment of cardiovascular and stroke risk of anti-diabetic drugs should be continued.

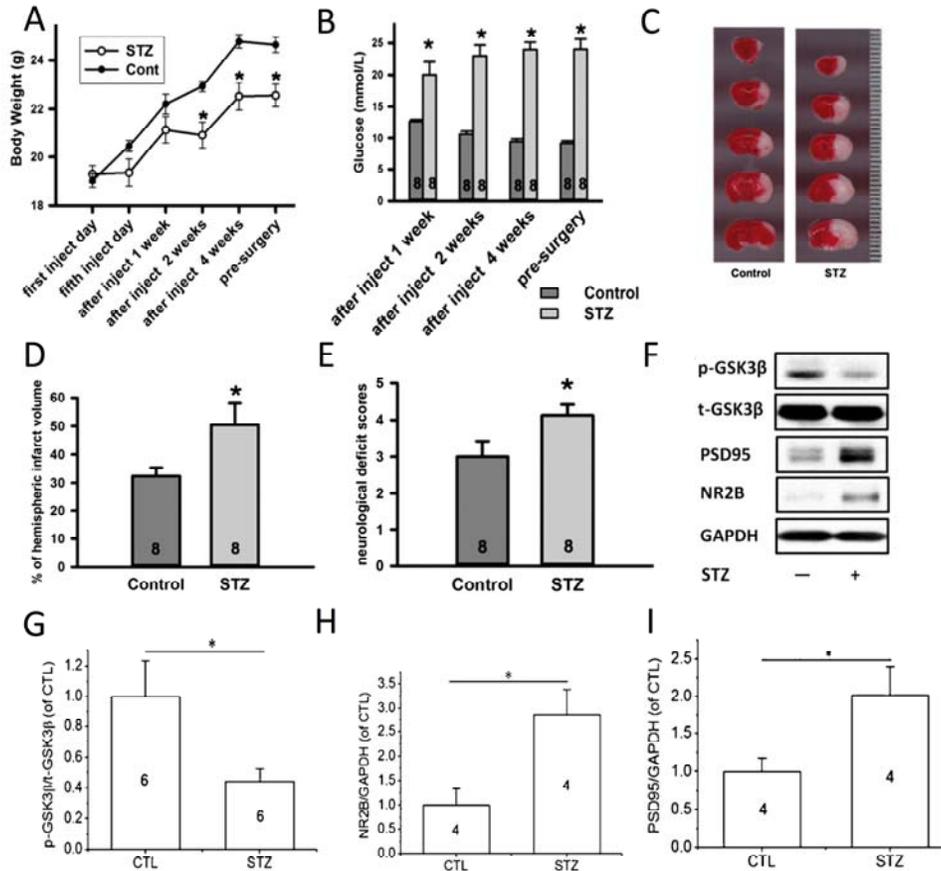


Figure 1. Effects of STZ-induced diabetes in increasing infarct volume in the mouse tMCAO model and molecules involved in the destructive effect of STZ-induced diabetes in mice. Body weights (A) and blood glucose (B) of mice before and 1, 2, 4, and 5 weeks after STZ or vehicle injections. Mean body weight was significantly reduced in animals 2 weeks after STZ injection compared with the controls. * $P < 0.05$. The blood glucose level in STZ-injected mice is significantly higher than that of the control animals, indicating successful induction of the diabetic mouse model. Hyperglycemia (≥ 16 mmol/L, Glucometer, Roche Diagnostics GmbH)¹⁴. * $P < 0.01$. (C) Representative images of TTC staining show that normal brain tissue is stained red and the infarcted brain tissue is stained white. (D) The effects of STZ-induced diabetes on brain infarct volume are shown compared with that of the vehicle-treated control animals. Mice with STZ-induced diabetes showed increased brain damage after the tMCAO compared with the vehicle-treated control group ($n = 8$ per group). * $P = 0.046$ by Student t test. (E) The 6-point standard scale is used for neurological deficit score in mice 24 h after tMCAO. The STZ-administered mice with tMCAO exhibited significantly higher neurological deficit scores than the vehicle-treated group. * $P = 0.047$. (F) Representative Western blot images show the protein levels of NR2B, PSD95, p-GSK3b, and t-GSK3b from brain tissues from STZ-administered and vehicle-treated groups. (G) Relative levels of p-GSK3b vs. t-GSK3b were determined by densitometry of the blots. The ratio of p-GSK3b and t-GSK3b proteins was significantly decreased in the STZ-administered group compared with that of the vehicle-treated control (CTL) group ($n = 6$ per group). * $P < 0.05$. Relative levels of NR2B, PSD95, and GAPDH were determined by densitometry of the blots, and NR2B-to-GAPDH (H) and PSD95-to-GAPDH (I) ratios were calculated. Protein levels of NR2B and PSD95 in the brains were upregulated in the STZ-administered mice compared with the vehicle-treated group ($n = 4$ per group). * $P < 0.05$.

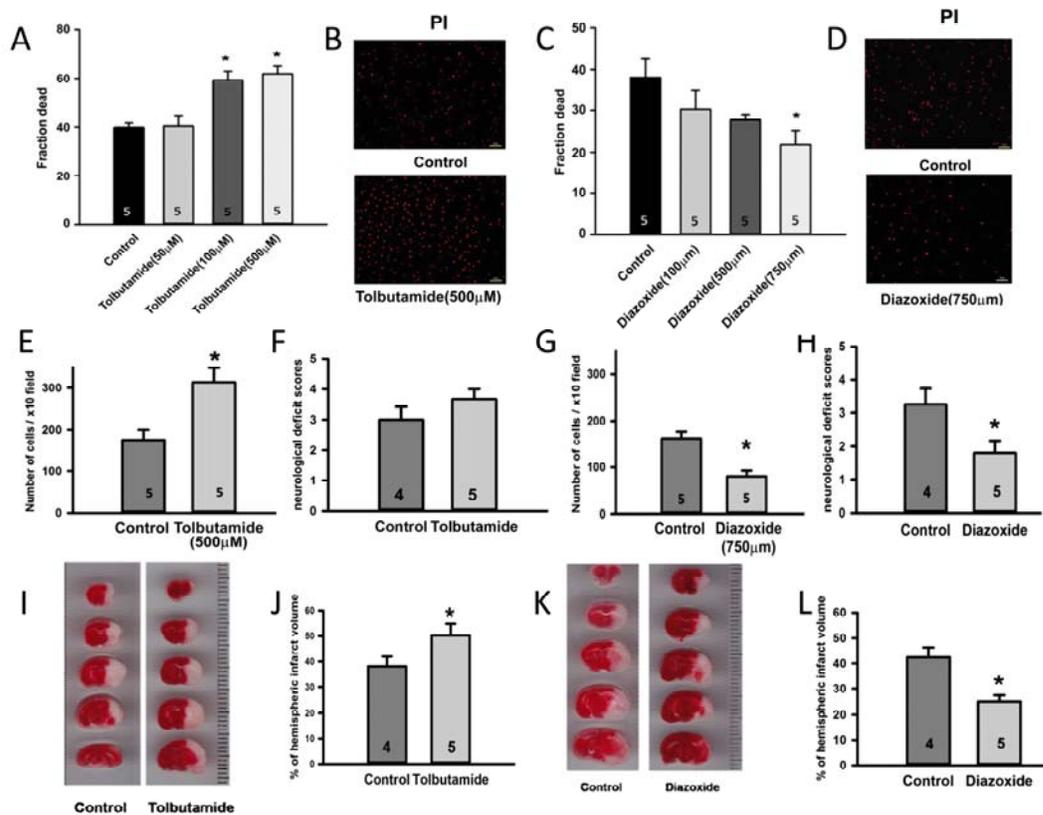


Figure 2. Effect of tolbutamide and diazoxide in OGD-induced neuronal cell injury and on infarct volume in mouse pMCAO model. Tolbutamide (50, 100, and 500 mmol/L) or DMSO (0.5%) (tolbutamide-vehicle-treated control group) or Diazoxide (100, 500, and 750 mmol/L) or 0.5N NaOH (Diazoxide-vehicle-treated control group) was applied 30 min before OGD. Cell damage was assessed 24 h after OGD. Quantitative measurements of PI fluorescence of cortical cultures 24 h after OGD showed that the fraction dead was significantly higher in the tolbutamide treated group (A) and significantly lower in diazoxide treated group (C) than in the control group. * $P < 0.05$. Confocal fluorescence imaging shows a significantly higher number of PI-positive cells in the tolbutamide-treated group (B) and lower number in diazoxide-treated group (D) than in the control group. (E and G) Quantitative analysis was performed by counting five random fields per coverslip ($n = 5$, with 5 slices included in each sample). Scale bars: 50 μm (x10 objective). The fraction of OGD-induced cells dead in each culture was calculated as: $\text{fraction dead} = (F_t - F_o) / (F_{\text{NMDA}} - F_o)$, where F_o is the initial fluorescence reading of the plate before OGD, F_t is the maximum fluorescence reading after OGD, and F_{NMDA} is the PI fluorescence of sister cultures 24 h after a 60 min exposure to NMDA (1 mmol/L) at 37°C¹⁵. The relative assessments of neuronal cell death were normalized by comparison with 100% cell death induced by NMDA. The result represents OGD-induced damage as a percentage of NMDA-induced cell death. (I and K) Representative TTC images. Effects of tolbutamide (J) and diazoxide (L) are shown on brain infarct volume compared with the vehicle-treated control animals. * $P < 0.05$ by Student t test. The 6-point standard scale was used for the neurological deficit score in mice 24 h after pMCAO. (F) The tolbutamide-treated mice ($n = 5$) with pMCAO exhibited higher neurological deficit scores than the vehicle-treated group ($n = 4$), but the difference was not statistically significant ($P = 0.193$). (H) The diazoxide-treated mice ($n = 5$) with pMCAO exhibited significantly lower neurological deficit scores compared with the vehicle-treated group ($n = 4$). * $P = 0.046$.

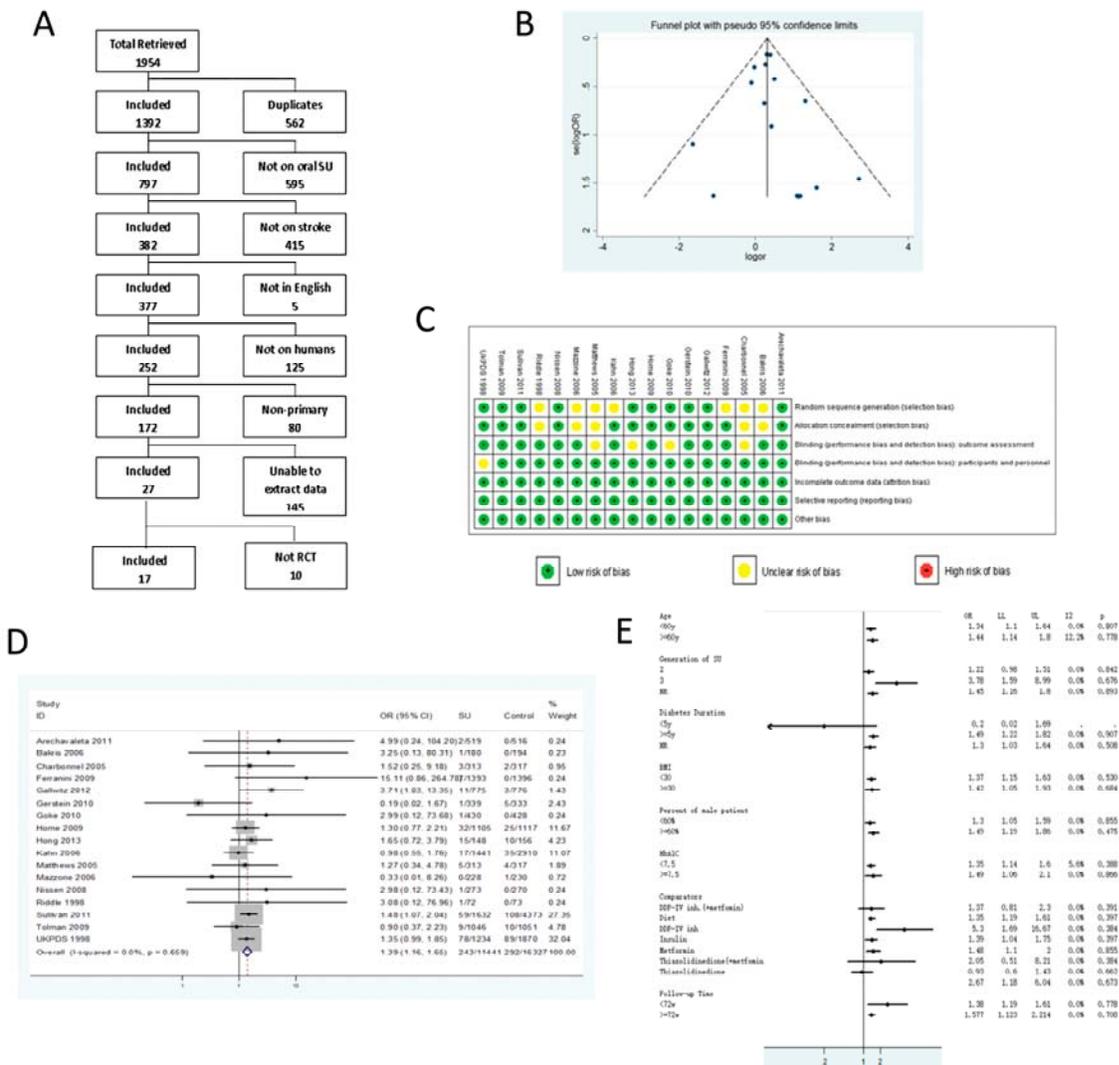


Figure 3. A systematic meta-analysis of 17 randomized control trials (RCT). (A) Study selection process. (B) Funnel plot for the 17 included trials to visualize potential publication bias. The shape of funnel plots did not reveal obvious evidence of asymmetry. (C) Quality of the included articles was assessed using “risk of bias” assessment tool from the Cochrane Library. The quality of the extracted studies was assessed by the two experimenters independently using the criteria for judging risk of bias as suggested by the Cochrane Handbook of Reviews of Assessing Risk of Bias. Disagreements were resolved by discussion between the two experimenters or the third person by referencing the original reports. (D) The individual and pooled OR with 95% CI of the incidence of stroke during treatment: any SU treatment (single use and combination) vs. any other treatment without SU. The pooled OR and 95% CI for the 17 trials were 1.39 (95% CI 1.16–1.65; P = 0.659). (E) Subgroup analyses of relative risks for incidence of stroke during treatment: any SU treatment (single use and combination) vs. any treatment without SU. Random effects analysis was used. DPP-IV inh, dipeptidyl peptidase-4 inhibitor; LL, lower limit; NR, not recorded; UL, upper limit. All randomized controlled trials comparing SUs and placebo or other anti-diabetics was retrieved. Results of unpublished trials were retrieved if they were available on www.clinicaltrials.gov, www.clinicalstudyresults.org, or www.controlled-trials.com. Multivariate logistic regression was used to determine overall risk of stroke occurrence with each anti-diabetic drug.

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