SSN-Hippocampus kuda protein

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Original article

Hippocampus kuda protein hydrolysate improves male reproductive dysfunction in diabetic rats

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ABSTRACT

The global prevalence of diabetes mellitus is rapidly increasing. This disease is associated with many complications including male reproductive dysfunctions and infertility. Seahorse (Hippocampus kuda) is a marine teleost fish well known for its beneficial effects on the reproductive system in traditional Chinese medicine books. Recently, several studies have been shown that the enzymatic hydrolysate of seahorse has multiple pharmacological activities. This study aimed to investigate the seahorse peptide hydrolysate (SH) ameliorative effects on the diabetic-induced male reproductive dysfunction in rat models. The in vivo studies were carried out with three different doses of SH (4, 8, and 20 mg/kg) and the diabetes condition was induced by administrating with streptozotocin (35 mg/kg) and fed a 40% high-fat diet. Seahorse hydrolysate (20 mg/kg) inhibited lipid peroxidation, increased antioxidant enzyme activity, and restored seminiferous tubules morphology in testis. Moreover, it improved reproductive dysfunction by increasing the level of testosterone, follicle-stimulating hormone, luteinizing hormone, sperm count, and motility. According to these results, we suggested that SH exhibited amelioration effects on the reproductive dysfunction.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia caused by the inability of the cells to utilize glucose properly. Type 1 and type 2 DM are the most common types of DM. Type 1 DM is characterized by the deficiency of insulin, whereas type 2 DM is peripheral insulin resistance [1]. The global prevalence of DM is rapidly increasing as a result of aging and lifestyle changes. International Diabetes Federation (IDF) estimated that 451 million people around the world were suffering from diabetes in 2017 and it is expected to reach 693 million in 2045 [2]. Whereas, type 2 DM is more prevalent than type 1 DM [3]. In diabetes, oxidative stress is occurring due to the overproduction of oxygen free radicals and the reduction of antioxidant defenses. The diabetes complications include retinopathy, neuropathy, nephropathy, cardiovascular diseases, and male reproductive dysfunction [4]. A previous study showed that DM has an adverse effect on male reproductive function such as structural changes in the testis, impairment of sperm parameters, and hormonal alterations in the hypothalamus-pituitary-gonadal (HPG) axis that finally results in infertility [5]. Additionally, recent studies also reported that DM conditions in the rat models affect vital organs including reproductive organs and resulting in testicular dysfunction. The reduction of testes index and weight, as well as sperm count and motility, was also observed in the DM group [6,7]. Several antidiabetic agents have been used to treated DM and its complications including testicular damage, however, most of these agents are not effective [7]. Therefore, novel effective antidiabetic agent's investigation with fewer adverse effects is a major challenge in future research.

Seahorse (*Hippocampus kuda*) is a marine teleost fish well known for its unusual characteristic feature and its unique medicinal properties and it has been used for thousands of years as an important and precious traditional Chinese medicine. Seahorses mainly occupy both temperate and tropical coastal water with a latitudinal distribution from about 50° North to 50° South [8]. The biofunctional compounds reported from seahorse predominantly include steroids, minerals, unsaturated fatty acids, and protein [9]. According to the traditional Chinese medicine book, the seahorse has beneficial effects on the regulation of urinogenital, reproductive, nervous, endocrine, and immune systems. Also,

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recent pharmacological studies suggested that the seahorse has multiple biological activities, including anti-tumor, anti-inflammatory, anti-oxidative, and neuroprotective activities [10].

Recently, many studies have shown that enzymatic hydrolysate from seahorse has a positive effect on health due to the bioactive peptides. A study reported that the peptide isolated from seahorse by enzymatic hydrolysis has potential beneficial effects in both osteoblastic MG-63 and chondrosarcoma SW-1353 cells [11]. Alcalase and pepsin hydrolysates from seahorses can improve the serum testosterone level in male ICR mice [12]. According to these conditions, we hypothesized that enzymatic hydrolysates from seahorse (*Hippocampus kuda*) can improve male reproductive dysfunction in an experimental diabetic model. Therefore, this study aimed to investigate the ameliorative effects of seahorse hydrolysates on male reproductive dysfunction in diabetic rat models.

2. Materials and methods

2.1. Materials

Dried seahorse (*Hippocampus kuda*) was provided by Longwalk Marine Biotech Co., Ltd (Kaohsiung, Taiwan). Alcalase (P4860), O-Phthalaldehyde (OPA), Leucine-Glycine (Leu-Gly), 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), sodium nitrite, streptozotocin (STZ), and nitro blue tetrazolium (NBT) were purchased from Sigma (St. Louis, MO, USA). Metformin was purchased from TCM Biotech International Corp. (Taipei, Taiwan). Dulbecco's modified eagle's medium, nutrient mixture F12 (DMEM-F12), fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640, and trypsin were purchased from Gibco (Carlsbad, CA, USA). Formalin was purchased from Macron Fine Chemicals (Center Valley, PA, USA). The glucose enzymatic kit and enzymatic antioxidant (superoxide dismutase, glutathione peroxidase, catalase, glutathione) commercial kits were purchased from Randox Laboratories (Crumlin, UK). The insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Interlab Co., Ltd (Taipei, Taiwan). The IL-1β ELISA kit, IL-6 ELISA kit, and TNF-α ELISA kits were purchased from Elabscience (Wuhan, China). The testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) ELISA kits were purchased from Fine Biotech Co., Ltd (Wuhan, China).

2.2. Seahorse hydrolysate preparation

Five grams of dried seahorse powder were mixed with 100 mL of distilled water and hydrolyzed with alcalase at 50 °C for 24 h. The enzyme to substrate ratio was 1:100 (w/w). At the end of 24 h, the hydrolysates were heated in boiling water for 10 min to inactivate the enzyme. Afterward, the seahorse hydrolysate (SH) was dried using a freeze dryer and stored at −20 °C for animal experiment and further use [12].

2.3. Protein content measurement

The protein content was measured by Bradford assay. A standard curve was established by serial dilution of bovine serum albumin (BSA) (1 mg/mL) with distilled water. Seahorse hydrolysate samples and BSA standard were adjusted to 20 μL, combined with 200 μL of Bradford reagent and incubated at room temperature for 15 min. The absorbance was then measured at 595 nm using the ELISA reader [13].

2.4. Degree of hydrolysis measurement

The degree of hydrolysis was measured by O-Phthaldialdehyde (OPA) assay. A standard curve was established by serial dilution of leucine-glycine (Leu-Gly) with distilled water. Seahorse hydrolysate samples and standard were added to wells in a 96-well microplate and combined with an OPA reagent mixture. Samples were incubated at room temperature for 2 min. The absorbance was then measured at 340 nm using the ELISA reader [14].

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Total protein from seahorse hydrolysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 1 mm thick, 12% polyacrylamide gel. Following SDS-PAGE, separated proteins were stained with Coomassie blue according to the previous method [15].

2.6. Animal experiment

The Institutional Animal Care and Use Committee (IACUC Approval No. 108018) of the National Taiwan Ocean University reviewed and approved all protocols. Briefly, five-week-old Sprague-Dawley rats were purchased from BioLASCO Taiwan Co., Ltd (Taipei, Taiwan). The rats were maintained under standard laboratory conditions (temperature 23 ± 1 °C, humidity 40–60%, 12 h light/dark cycle). Forty-two rats were housed individually and provided a standard chow-fed diet (LabDiet 5001) and water ad libitum. After the 1-week acclimatization phase, the rats were divided into Control (Con) group ($n = 7$) and the Diabetic group ($n = 35$) as shown in Fig. 1. The Con group was fed with a standard chow-fed diet and the Diabetic group was fed with the high-fat diet (40% calories made by lard) for 3 weeks. After 3 weeks of feeding, the Diabetic group was injected with a low dose of streptozotocin (STZ, 35 mg/kg) to induce type 2 diabetes condition in the rats [16]. After 1-week induction of STZ, the oral glucose tolerance test (OGTT) was performed to evaluate blood glucose levels. The rats were confirmed as diabetes if the fasting blood glucose (FBG) ≥ 126 mg/dL or the glucose concentration ≥ 200 mg/dL at 2-h post-load glucose [17,18]. Then, the diabetic rats were divided into five groups ($n = 7$) and there is no animal death during the experiment. The minimum animal number of each group was considered from previous studies [19–21]. The first group was diabetes rats without any treatment (DM) and the other groups were oral gavage treated with either one of three different doses of seahorse hydrolysate (SH1: 4 mg/kg, SH2: 8 mg/kg, SH5: 20 mg/kg) or metformin (Met: 200 mg/kg). The daily total volume of the SH or Met administrations during treatment depended on the rats' bodyweight of each dosage [22]. Rats were orally administered for 6 weeks and then OGTT was conducted before sacrificed. The extract dose and duration of the treatment were chosen according to previous methods [23,24]. The rats have fasted for 12 h before sacrifice. It was sacrificed in an empty chamber by exposure to CO_2 until euthanasia [25,26]. The epididymis and epididymis fat were separated and weighted in a digital balance. The sperm from the epididymis was collected and evaluated immediately. One of the testes was kept at −80 °C and another one was fixed in 10% neutral buffered formalin solution for further analysis.

2.6.1. Plasma collection and testis tissue homogenization

On the day of sacrifice, the whole blood was collected from the rats then centrifuged at 3000 rpm for 15 min at 4 °C to separate the plasma. The supernatant (plasma) was collected and kept for future analysis [27].

One of the testes was stored at −80 °C. After freezing, testis tissue slices were taken and washed with phosphate-buffered saline (PBS). Tissue was suspended in cold PBS and homogenized by using a micro-tube homogenizer. Then, they were frozen and thawed twice. Afterward, they were centrifuged at 5000 rpm for 15 min. The supernatant was collected and stored at −80 °C [28].

2.6.2. Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was carried out by orally administering glucose (2 g/kg) after 12 h of fasting and blood was drawn to measure glucose levels at 0, 30, 90, and 120 min after glucose

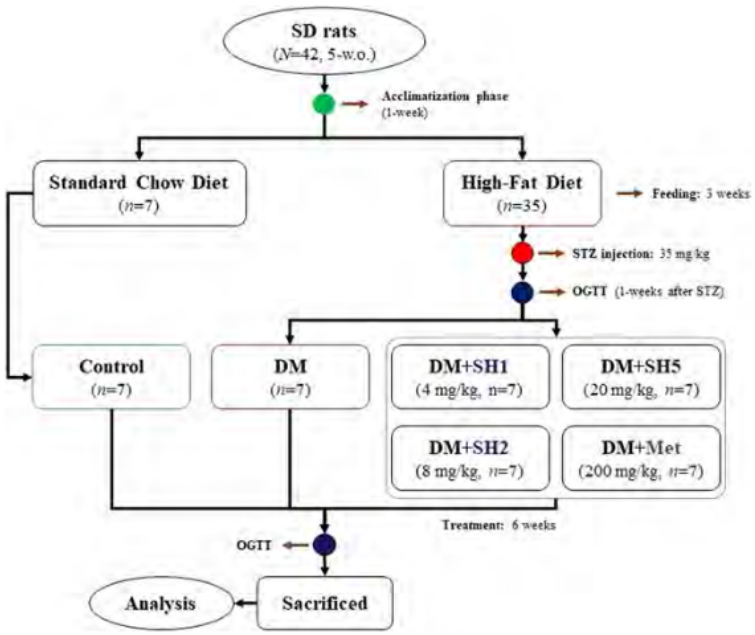


Fig. 1. The flowchart of seahorse hydrolysate treatment on male reproductive dysfunction in diabetic rats. DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin; OGTT: Oral glucose tolerance test; STZ: Streptozotocin.

injection according to a previous method [29]. The plasma glucose concentration was determined by a glucose enzymatic kit (Randox, Crumlin, UK).

2.6.3. Insulin, proinflammatory cytokines, and hormone analysis

Insulin, tumor necrosis factor-α, interleukin (IL)−6, IL-1β, follicle-stimulating hormone, luteinizing hormone, and testosterone were analyzed by using enzyme-linked immunosorbent assays (ELISA) kits according to manufacturer's protocols. The homeostasis model assessment-insulin resistance (HOMA-IR) level was calculated by using the following formula: fasting plasma insulin (mU/mL) multiplied by fasting plasma glucose (mmol/L)/22.5 [30].

2.6.4. Oxidative stress analysis

The superoxide dismutase, glutathione peroxidase, glutathione, and catalase antioxidant activities in testis were analyzed by using the Randox kit. Whereas, malondialdehyde (MDA) was used to evaluate lipid peroxidation and evaluated according to a previously reported method [31]. Briefly, testis homogenate was mixed with reagent (15% trichloroacetic acid in 0.25 N hydrochloric acid (HCl) and 0.375% thiobarbituric acid in 0.25 N HCl) and placed in a water bath at 100C for 15 min. After cooling, 300 μL n-butanol was added and centrifuged at 1500*g* for 10 min. The supernatant was measured at the 532 nm absorbance.

For determining MDA content in sperm, sperm (10^6 cells/mL) was added to the reagent and then followed the same procedure as mentioned above. Nitric oxide (NO) assay was conducted by using Griess reaction according to previous reference [32]. Whereas, superoxide production (NBT assay) was estimated by following the previous method [33]. Briefly, the number of sperm cells was adjusted to 10^6 cells/mL. Then, NBT solution [0.1 mg/mL NBT, 5% FBS, and 3% dimethyl sulfoxide (DMSO) dissolved in 10 mL DMEM] was added and incubated at 37 °C in 5% CO_2 for 1 h. After centrifugation at 500*g* for 10 min, the supernatant was removed and 200 μL DMSO was added and shaken for 5 min. The absorbance at 570 nm was measured by an ELISA reader.

2.6.5. Sperm parameters

Sperm were prepared by using the swim-up technique from epididymis tissue according to the previous method [34]. Briefly, the epididymal tissues were cut in the RPMI medium and shaken at 100 rpm for 10 min. After that, they were transferred into the tube and centrifuged at 100*g* and then incubated at 37 °C in a 5% CO_2 incubator for 30 min. Finally, the sperm cells were collected from the supernatant and pipetted to a hemocytometer, then observed under a light microscope with a computer screen-assisted to observe sperm count, motility, and abnormalities.

2.6.6. Testis histopathological

One of the testes was fixed in 10% neutral buffered formalin [35]. Then, the tissues were sliced into the micro size and attached to the slide, and then send to Rapid Science Co., Ltd (Taichung, Taiwan) for hematoxylin and eosin stain (H&E) staining.

2.7. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses by one-way analyses of variance (ANOVA) with Duncan Post-Hoc test ($p < 0.05$) were conducted using SPSS 22.0 (IBM Corporation, Armonk, NY, USA) software to analyze the experimental data.

3. Results

3.1. Protein content and degree of hydrolysis

The protein content of the seahorse hydrolysate (SH) was about 6.23 ± 1.07 mg BSA equivalent/g dried sample with 29.04 ± 1.75% of the degree of hydrolysis. It demonstrated that protein was broken down into peptides. Also, it could be proved by checking the protein molecule's weight by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The two major protein bands of SH were found at 63 and 33 kDa (Fig. 2).

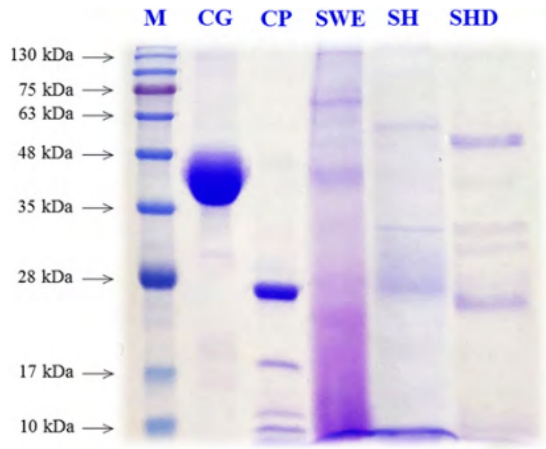


Fig. 2. SDS-PAGE profile of seahorse hydrolysate. M: protein marker; CP: control protein (trypsin); CG: control glycoprotein (horseradish peroxidase); SWE: seahorse water extract; SH: seahorse hydrolysate; SHD: seahorse hydrolysate GI digest.

3.2. Seahorse hydrolysate improves fasting glucose, insulin, and homeostasis model assessment-insulin resistance levels

The blood glucose dan area under the curve of the DM group before SH treatment was significantly ($p < 0.01$) higher than the Con group as shown in Fig. 3A and B. After 6 weeks of treatment, a high level of fasting plasma glucose was observed in untreated diabetes (DM) group. This value significantly different ($p < 0.05$) when compared to the Control (Con) and Metformin (Met) groups (Fig. 3C and D; Table 1). On the other hand, the SH-treated group reduced glucose with no significant difference ($p > 0.05$) with DM and Met groups. However, high-dose of SH (DM+SH5) treatment significantly ($p < 0.05$) decreased the homeostasis model assessment-insulin resistance (HOMA-IR) values. As a positive control, Metformin treatment significantly reduced the HOMA-IR level.

3.3. Seahorse hydrolysate reduces proinflammation cytokines levels

Fig. 4 showed an elevated expression of plasma interleukin (IL)−6, IL-1β, and tumor necrosis factor (TNF)-α. These conditions significantly ($p < 0.05$) higher when compared to the Con group. After treatment with SH and Met, the IL-6 expression was significantly reduced (Fig. 4A). The IL-1β level also significantly ($p < 0.05$) reduced by SH and Met treatment. Whereas, high-dose of SH (DM+SH5) showed a more significant reduction effect when compared to other groups (Fig. 4B). Additionally, a high dose of SH treatment was observed significantly ($p < 0.05$) decreased TNF-α level (Fig. 4C).

3.4. Seahorse hydrolysate enhances antioxidant activities and suppresses oxidative stress

Reduction of some enzymatic antioxidant activities was observed in the DM group (Fig. 4). Superoxide dismutase (SOD) activity significantly decreased in the DM group and this level significantly ($p < 0.05$) improved by SH and Met treatment (Fig. 5A). Whereas, a high dose of SH treatment also significantly ($p < 0.05$) enhanced catalase and glutathione peroxidase (GPx) activities (Fig. 5B and C). Glutathione (GSH) activity also was observed ($p < 0.05$) significantly increased after treatment with medium and high doses of SH (Fig. 5D).

Some oxidative stress markers also were observed in this present study (Fig. 6). The malondialdehyde (MDA) level was significantly ($p < 0.05$) reduced after 6 weeks of treatment with SH and Met (Fig. 6A). These treatments also significantly ($p < 0.05$) decreased superoxide anion (O_2^-) production which indicated by low nitro blue

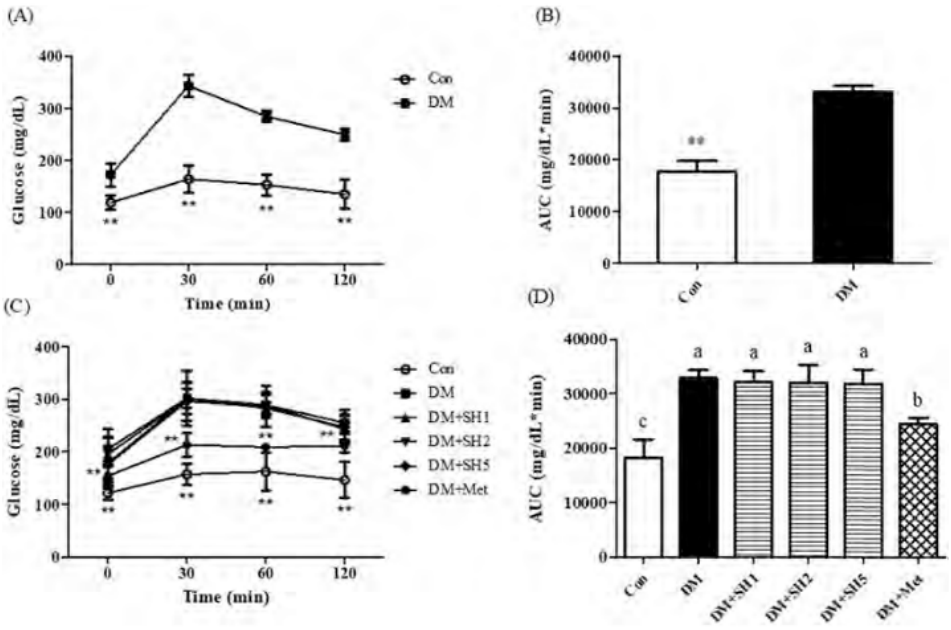


Fig. 3. The oral glucose tolerance test (OGTT) and the area under curve (AUC) of plasma glucose concentration during oral glucose tolerance test in diabetic rats. (A-B) plasma glucose before SH treatment. Data are shown as the mean ± SD (Con group, $n = 7$; DM group, $n = 35$). (C-D) plasma glucose after SH treatment. Data are shown as the mean ± SD ($n = 7$). * $p < 0.05$ or ** $p < 0.01$ vs DM group. The values with different letters (a-b) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test. Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin.

Table 1
Fasting plasma glucose level, plasma insulin level, and homeostasis model assessment-insulin resistance (HOMA-IR) in diabetic rats after treatment of SH.

Properties	Con	DM	DM+SH1	DM+SH2	DM+SH5	DM+Met
Fasting Glucose (mg/dL)	121.78 ± 12.90^{c}	205.63 ± 23.58^{a}	195.91 ± 47.47^{ab}	178.39 ± 47.65^{ab}	176.36 ± 32.91^{ab}	150.13 ± 17.70^{bc}
Insulin (μg/L)	0.26 ± 0.08^{bc}	0.34 ± 0.03^{a}	0.30 ± 0.01^{ab}	0.30 ± 0.01^{ab}	0.24 ± 0.07^{c}	0.27 ± 0.02^{bc}
HOMA-IR	1.66 ± 0.52^{d}	3.68 ± 0.67^{a}	3.12 ± 0.75^{ab}	2.80 ± 0.75^{bc}	2.21 ± 0.79^{cd}	2.20 ± 0.22^{cd}

Data are shown as the mean ± SD (n = 7). Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin. The values with different letters (a-d) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test.

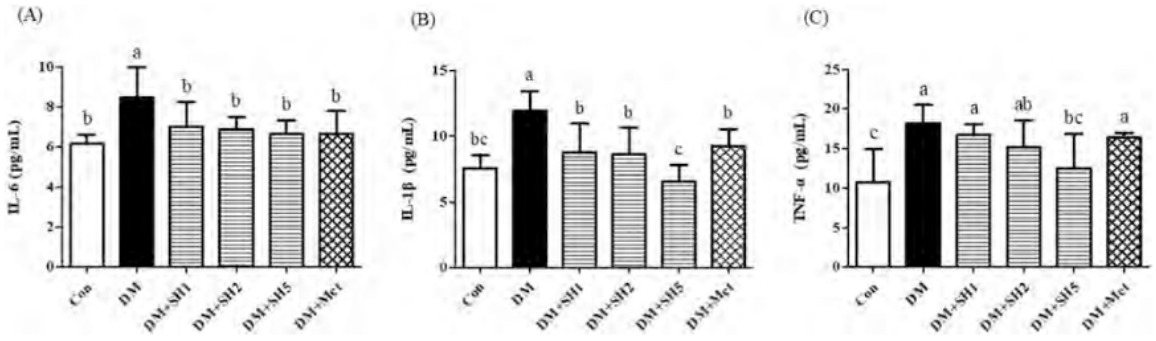


Fig. 4. Effects of seahorse hydrolysate on plasma proinflammatory cytokine levels: (A) interleukin-6 (IL-6), (B) interleukin-1 beta (IL-1β), and (C) tumor necrosis factor-alpha (TNF-α) levels in diabetic rats after treatment. The values with different letters (a-c) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test. Data are shown as the mean ± SD ($n = 7$). Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin.

tetrazolium (NBT) reduction as shown in Fig. 6B. Nitric oxide (NO) level also was observed significantly ($p < 0.05$) decreased after treatment by SH and Met (Fig. 6C).

3.5. Effects of seahorse hydrolysate on testis, epididymis, and epididymal fat weights

As shown in Table 2, the testis and epididymis weights decreased in the DM group. These values significantly different ($p < 0.05$) when compared to SH and Met groups. Whereas, SH and Met treatment significantly ($p < 0.05$) protected the reduction of testis and epididymis weights. On the other hand, SH and Met treatment significantly ($p < 0.05$) decreased epididymal fat.

3.6. Seahorse hydrolysate elevates testosterone, follicle-stimulating hormone, and luteinizing hormone expressions

Low plasma testosterone level was observed in the DM group (Fig. 7A). This level significantly ($p < 0.05$) increased when compared to the DM group after treatment with SH and Metformin for 6 weeks. Medium- and high-doses of SH treatment also significantly ($p < 0.05$) elevated follicle-stimulating hormone levels in diabetic rats (Fig. 7B). Additionally, a low level of luteinizing hormone (LH) was observed significantly ($p < 0.05$) reduced in the DM group and this level significantly ($p < 0.05$) increased after 6 weeks of treatment with SH and Metformin (Fig. 7C).

3.7. Effects of seahorse hydrolysate on sperm properties and seminiferous tubules of rat testis morphology

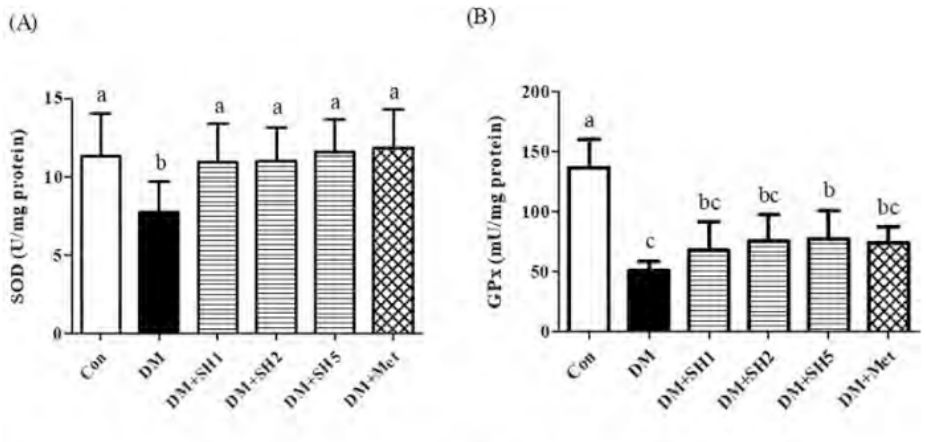
Total sperm count significantly ($p < 0.05$) lower in the DM group when compared to the Con group as shown in Fig. 8A. Sperm motility was observed significantly ($p < 0.05$) decreased in the DM group (Fig. 8B). Additionally, sperm abnormalities significantly ($p < 0.05$) increased in the DM group (Fig. 8C). The sperm motility was significantly ($p < 0.05$) increased after treatment with a high dose of SH. Whereas, the sperm abnormalities was significantly ($p < 0.05$) reduced by treating with SH. As a positive control, metformin treatment significantly ($p < 0.05$) increased total sperm count and sperm motility as well as reduced sperm abnormalities. As shown in Fig. 9, the structure of seminiferous tubules of the testis of streptozotocin-induced diabetic rats separated from each other and appeared shrunken (Fig. 9B). However, this structure successfully improved after treated with medium- and high-doses of SH for 6 weeks as shown in Fig. 9E and F, respectively.

4. Discussion

In this present study, we have demonstrated the ameliorative effects of protein hydrolysate from seahorse (*Hippocampus kuda*) on male reproductive dysfunction in a diabetic rat model. The seahorse protein hydrolysates (SH) samples were obtained by hydrolysis with alcalase enzyme. Recently, many studies have shown that enzymatic hydrolysate from seahorse has a positive effect on health due to the bioactive peptides. Alcalase and pepsin hydrolysates from seahorses can improve the serum testosterone level in male ICR mice [12].

The diabetic condition was induced by feeding with a high-fat diet and intraperitoneal injection of a low dose of streptozotocin. A previous reference reported that this condition is suitable to model for type 2 diabetes mellitus (T2DM) [16]. Hyperinsulinemia and hyperglycemia are the classical key factors of T2DM. The hyperglycemia condition can induce oxidative stress by several mechanisms, such as glucose autoxidation, polyol pathway, and advanced glycation end-products (AGE) formation [36]. Results indicated that the untreated diabetic (DM) group showed an increased fasting plasma glucose in comparison with the Control (Con) group and metformin (Met) group. Also, the insulin level of the DM group was showed higher than Con and high-dose of SH treated groups (Table 1). The HOMA-IR has been widely used for the estimation of insulin resistance (IR). A previous study showed that oxidative stress enhances IR in rat models [37]. In this study, high-dose of SH and Met groups showed that the HOMA-IR level was lower when compared with the DM group.

Excessive glucose induces the inflammation effect through oxidative



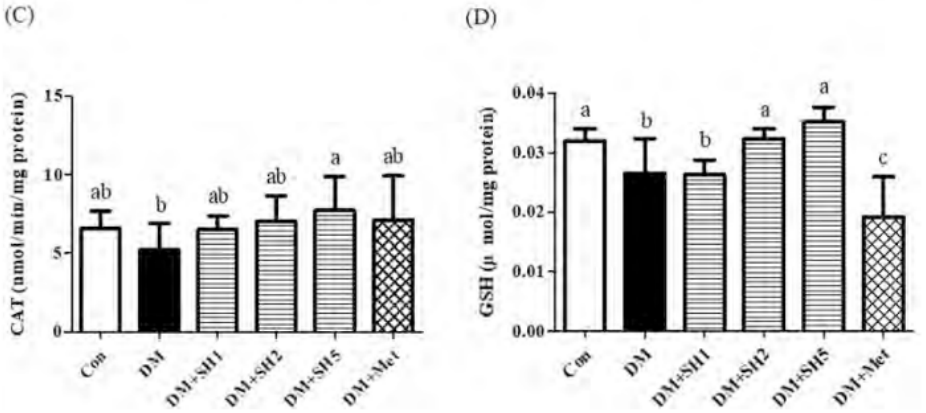


Fig. 5. Effects of seahorse hydrolysate on enzymatic antioxidant activities: (A) superoxide dismutase (SOD), (B) glutathione peroxidase (GPx), (C) catalase (CAT), and (D) glutathione (GSH) in testis of diabetic rats after treatment of seahorse hydrolysate (SH). Data are shown as the mean ± SD ($n = 7$). The values with different letters (a-c) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test. Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin.

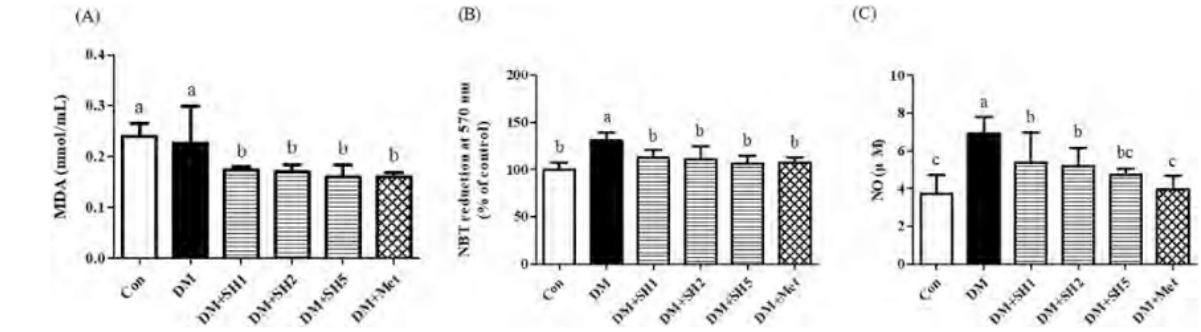


Fig. 6. Effects of seahorse hydrolysate on oxidative stress markers: (A) malondialdehyde (MDA), (B) superoxide anion production (NBT reduction), and (C) nitric oxide (NO) levels in serum of diabetic rats after treatment. The values with different letters (a-c) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test. Data are shown as the mean ± SD ($n = 7$). Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin.

stress and reduced antioxidants [38]. The adipose tissue, liver, muscle, and pancreas are the sites of inflammation in the presence of T2DM. Macrophages into these tissues are infiltrated by mononuclear cells and are in a state of chronic inflammation in animal models of diabetes. The infiltrated macrophages secrete proinflammatory cytokines, such as tumor necrosis (TNF)-α, interleukin (IL)−6, and resistin [39]. Results indicated that the IL-6 and IL-1β expression levels in plasma were lower in Con and SH-treated groups, and high-dose of SH group showed significantly decreased plasma TNF-α production. The DM group was accompanied by the overexpression of IL-6, IL-1β, and TNF-α (Fig. 4).

The enzymatic antioxidant activities including superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (Cat), and glutathione (GSH) of the DM group significantly decreased in testis. After treated with seahorse hydrolysate significantly increased these expressions (Fig. 5). A previous study reported that oxidative stress has been recognized as one of the risk factors of male reproductive dysfunction [5]. According to these results, seahorse hydrolysate might improve reproductive function by alleviating oxidative stress and enhancing

Table 2
The testis, epididymis, and epididymal fat weights in diabetic rats after treatment of SH.

% of body weight	Con	DM	DM+SH1	DM+SH2	DM+SH5	DM+Met
Testis	0.71 ± 0.04^{bc}	0.67 ± 0.06^{c}	0.74 ± 0.05^{ab}	0.76 ± 0.09^{ab}	0.81 ± 0.07^{a}	0.81 ± 0.04^{a}
Epididymis	0.32 ± 0.03^{ab}	0.29 ± 0.04^{b}	0.32 ± 0.02^{ab}	0.33 ± 0.02^{a}	0.33 ± 0.02^{a}	0.35 ± 0.03^{a}
Epididymal fat	1.18 ± 0.08^{c}	2.03 ± 0.31^{a}	1.52 ± 0.41^{b}	1.41 ± 0.19^{bc}	1.31 ± 0.22^{bc}	1.39 ± 0.16^{bc}

Data are shown as the mean ± SD (n = 7). Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin. The values with different letters (a-c) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test.

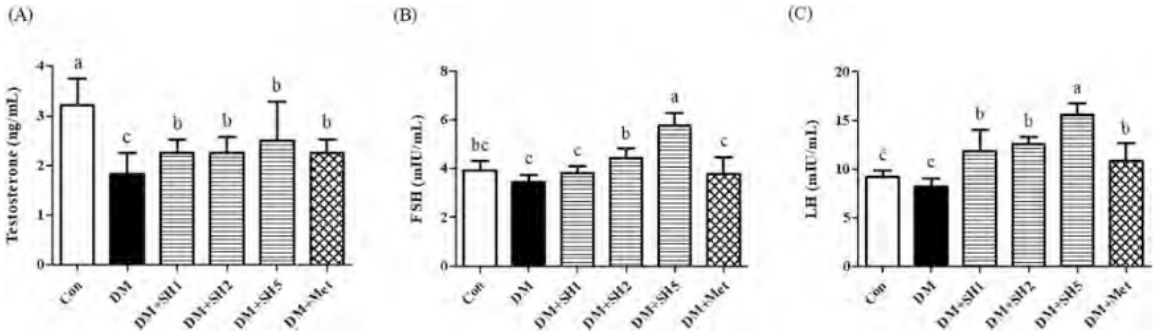


Fig. 7. Effects of seahorse hydrolysate on plasma (A) testosterone, (B) follicle-stimulating hormone (FSH), and (C) luteinizing hormone (LH) levels of diabetic rats after treatment. The values with different letters (a-c) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test. Data are shown as the mean ± SD ($n = 7$). Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin.

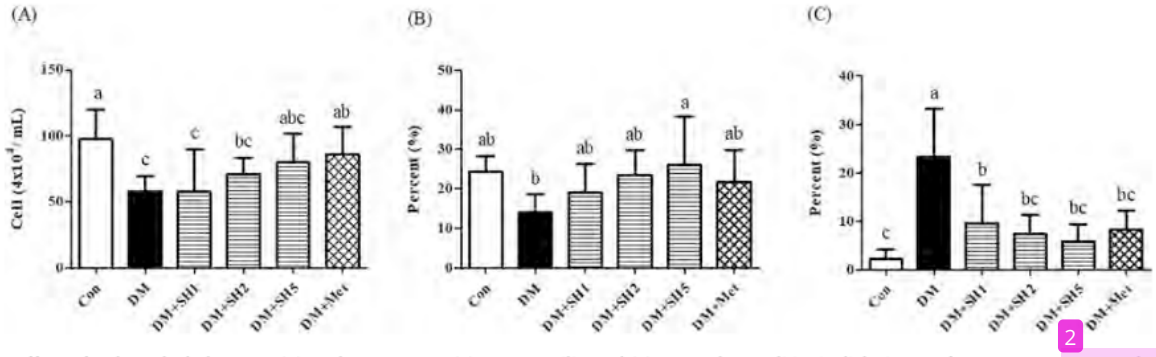


Fig. 8. Effects of seahorse hydrolysate on (A) total sperm count, (B) sperm motility and (C) sperm abnormalities in diabetic rats after treatment. Data are shown as the mean ± SD ($n = 7$). The values with different letters (a-c) represent significantly different ($p < 0.05$) as analyzed by Duncan's multiple range test. Con: control; DM: diabetes mellitus; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH; DM+Met: diabetes +200 mg/kg per day metformin.

enzymatic antioxidant activities.

Oxidative stress is characterized by increased reactive oxygen species (ROS) production. Oxidative damage to macromolecules including carbohydrates, proteins, and lipids are typically viewed as cellular damage to cause irreversible macromolecules modifications. Thus, the by-products of these oxidatively modified biomolecules are used as oxidative stress biomarkers such as malondialdehyde (MDA). Also, oxidative stress impairs the endogenous antioxidant defense system [40]. As shown in Fig. 6, treated with seahorse hydrolysate reduced MDA level especially in a dose-dependent manner.

Table 2 shows the testis, epididymis, and epididymal fat weights in the experimental groups. The testicular and epididymal weights were significantly increased in the SH-treated group when compared to the DM group. Furthermore, there were significant differences between the high-dose of SH group and the Con group. Testis weight is dependent on the mass of the differentiated spermatogenic cells therefore the reduction in the testis weight may be due to the decreased density of germ cells and spermatogenic arrest [41].

The hypothalamus-pituitary-gonadal (HPG) axis is finely regulated by the activity of GnRH neurons, a peculiar hypothalamic neuronal subpopulation, scattering within the hypothalamus [42]. Acting primarily in the anterior pituitary, GnRH binds to its receptor on the cell surface of the gonadotrope and stimulating the production of these gonadotropins. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) exert their effects on the testis, leading to steroidogenesis, and spermatogenesis [43]. Derangements of the HPG axis are often associated with metabolic disorders. The patients with T2DM displayed a low concentration of FSH and LH, indicating hypothalamic defects and impaired pituitary response to GnRH [44]. Fig. 7 revealed that plasma testosterone, FSH, and LH levels decreased in the DM group compared with the Con group. After treated with medium and high doses of SH, the levels of testosterone, FSH, and LH were considerably increased.

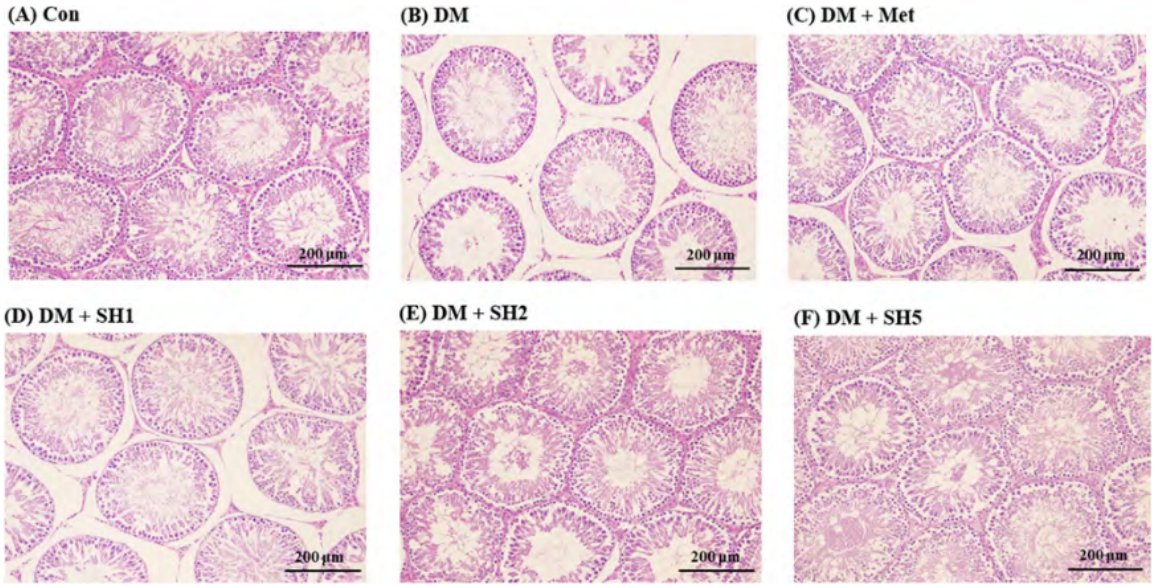


Fig. 9. Effects of seahorse hydrolysate on the morphology of seminiferous tubules of the diabetic rat testis after treatment. Testis were stained with hematoxylin and eosin stain (H&E). Con: control; DM: diabetes mellitus; DM+Met: diabetes +200 mg/kg per day metformin; DM+SH1: diabetes +4 mg/kg per day SH; DM+SH2: diabetes +8 mg/kg per day SH; DM+SH5: diabetes +20 mg/kg per day SH.

There are studies that described that STZ-induced DM male rats have diminished reproductive organ weight, testicular sperm content, epididymal sperm content, as well as sperm motility, and DM also causes regression of epididymis, leading to a decrease in caput weight, corpus, and caudal regions [45]. The accumulation of visceral fat is associated with hyperglycemia and oxidative stress. As shown in a previous study, obese mice were significantly higher oxidative stress levels in circulation [46]. Results showed that oral treatment of SH significantly decreased the epididymal fat weight when compared to in the DM group.

Sperm are made up of high polyunsaturated fatty acid in composition, which makes them sensitive to oxidative damage, and DM with sustained uncontrolled hyperglycemia is responsible for oxidative stress, resulting in decreased fertility potential [45]. As shown in Fig. 6, Control and seahorse hydrolysate treatment in STZ-induced diabetic rats exhibited lower MDA, NBT, and NO levels in sperm compared with the DM group. Previous studies also reported that diabetic conditions increase lipid peroxidation (MDA) levels [47,48]. Additionally, nitric oxide level also elevates in the diabetic group [49,50]. Disruption of spermatozoa membrane matrix structure due to lipid peroxidation may contribute to the deterioration of membrane integrity thus leading to the increase in abnormal morphology of sperm. Additionally, damage of the membrane causes disruption to its fluidity, leading to motility loss [41]. High-dose of SH group had increased and decreased the sperm mobility and abnormalities, respectively (Fig. 8). The reduction of sperm count was consistent with the depletion in the epididymal weight. Results showed that the DM group decreased sperm count compared with the Con group. A previous study also reported that DM condition reduces sperm count when compared to the control group [51]. In this present study, treatment with metformin and seahorse hydrolysate recovered total sperm count.

The testis is consisting of seminiferous tubules which are formed by the Sertoli cells, and the function of seminiferous tubules is to provide structural support for the developing germinal cells. In most mammals, the process of spermatogenesis occurs within seminiferous tubules that release spermatozoa into a rete testis which is connected to the epididymis. In the interstitium of the testis between tubules are the Leydig cells responsible for the production of androgen [52]. Histopathological observation showed the normal histological structure of the seminiferous tubules and Leydig cell, as well as abundant germinal cells in the Con group. Whereas the testicular structure in the DM group displayed morphological alterations with the reduction in the number of Leydig cells, diameters of the seminiferous tubule, and significant atrophy and a cavity were seen in the lumen [53,54]. Additionally, a previous study also reported that diabetes conditions induce a reduction of the diameter of the seminiferous tubule [55]. Oral supplementation of high-dose of SH improved structure and tubular diameter in testis (Fig. 9).

5. Conclusion

Oral administration of seahorse protein hydrolysate (SH) shows beneficial effects in diabetic rats. SH increased enzymatic antioxidant activity and inhibited lipid peroxidation. Additionally, SH also improved seminiferous tubules morphology in the testis and enhanced the level of testosterone, follicle-stimulating hormone, luteinizing hormone, and total sperm count. Based on these results, we supposed that SH might ameliorate the male reproductive dysfunction by improving the endocrine system or suppressing oxidative stress.

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Declaration of conflicting interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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