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Research Article

Immunohistochemical Localization of Leptin and Ghrelin in Kidney Tissue of Capsaicin Administered Diabetic and Non-diabetic Rats

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Abstract

In this study, we investigated the effect of Capsaicin (CAP) on leptin and ghrelin expression in diabetic kidneys by immunohistochemical method. The subjects were separated into 5 groups: control, sham, diabetes, CAP, and D+CAP. Streptozotocin (45 mg/kg, single dose) was administered to each rat in the diabetes and D+CAP groups intraperitoneally. Rats with blood glucose levels of 200 mg/dL or higher were considered diabetic. Capsaicin (1 mg/kg) was administered subcutaneously to the rats in the CAP and D+CAP groups every day for 2 weeks. H&E, PAS, and Crossman's Triple staining were performed to examine the histology of the kidney. Leptin and ghrelin in the kidney were examined by immunohistochemical method. Histological examination revealed normal kidney tissue in the control, sham, and CAP groups. The diabetes group showed the glomerulus with scattered and narrowing of the Bowman's capsule. While leptin immunoreactivity was most intense in the diabetes group, it was observed the weakest in the CAP and D+CAP groups. While strong ghrelin immunoreactivity was seen in the control and sham groups, mild immunoreactivity was seen in the CAP group, and weak immunoreactivity was seen in the diabetes and D+CAP groups. In this study, we concluded that the application of CAP could prevent loss of appetite by reducing the leptin expression during diabetes, but could not protect ghrelin from adverse effects of diabetes.

Keywords: Capsaicin, Diabetes, Ghrelin, Immunohistochemical, Leptin

Introduction

Diabetes Mellitus (DM) is a metabolic disease characterized by insulin secretion disorder or increased insulin resistance. A diabetic patient has an increased blood glucose level because of both of these conditions [1]. DM is asymptomatic or characterized by clinical symptoms. These clinical symptoms, which include weight loss, polyuria, polydipsia, polyphagia, and weakness, affect many organs and systems [2].

Capsaicin (CAP) is a chemical compound of chili and regulates the spiciness of hot chili pepper, usually termed a hot pepper *(Capsicum annuum L.)* [3]. *Capsicum annuum* L. contains protein, ascorbic acid, thiamine, red carotenoids, iron, copper, manganese, magnesium, zing, oxalates, phosphorus, flavonoids, calcium, water and vitamins, alongside capsaicin [4,5]. CAP consists of a long hydrophobic carbon tail containing a polar amide group (C18H27NO3) and a benzene ring. It is a lipophilic, volatile, pungent, hydrophobic, colorless, and odorless white crystalline powder [5]. CAP increases lipid peroxidation which decreases the amount of adipose tissue, and serum triglycerides. CAP has also an effect to inhibit the glycogen metabolism in skeletal muscles [6].

Leptin, which is known as an anorexigenic hormone, has an appetite suppression. It does this function by connecting its receptors in the hypothalamus. There is a relationship between leptin levels and some diseases such as cardiovascular diseases, chronic kidney disease, and DM [7,8]. It has been understood that it plays a role in the regulation of all system functions because leptin receptors are almost found in all organs [9]. A kind of leptin resistance can occur with leptin receptor downregulation, and leptin does not work properly in this condition [10,11].

Ghrelin is an orexigenic hormone that increases appetite so it has the opposite effect of leptin [12]. Ghrelin is found in many organs such as the stomach, liver, hypothalamus, pancreas, and kidney [13]. The ghrelin receptor is also expressed in the kidneys [14]. Ghrelin stimulates insulin secretion in the pancreas [15]. While there is insulin resistance in DM, ghrelin upgrades insulin signaling [16].

The purpose of this study is to investigate the effect of CAP on leptin and ghrelin by immunohistochemical method and on the histological structure by histochemical method in diabetic and non-diabetic rat kidneys.

Material and Methods

Ethical Statement

We received approval from Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK: 2019-057) for the applications performed on rats in the study.

Animals and Experimental Design

The study was started after the rats were adapted to the environment for one week. After this process, 30 female Wistar albino rats (three months aged, weighing 200-220 g) were separated into 5 groups: control, sham, diabetes, CAP, and D+CAP. The control group was left untreated, only a citrate buffer (0.1 M, pH 4.5) was injected via i.p. into the rats in the sham group, CAP was injected into the rats in the CAP and D+CAP groups and Streptozotocin (STZ) was injected to the rats in diabetes and D+CAP groups. Experimental applications were performed at Kafkas University Experimental Animal Application and Research Center. Rats were kept in standard cages in a room that had 22±2ºC room temperature, and 12 h of light and dark cycle. They were fed *ad libitum* with a standard rat diet and water except 8 h before STZ injection and taking blood samples. The duration of the study lasted for 14 days and the study started with STZ injection which was accepted the day 0. After 14 days, cervical dislocation was applied to the rats under ether anesthesia, and kidney tissues were taken.

Preparation of STZ and Induction of Diabetes

STZ (Sigma S0130-100 MG) was dissolved in a citrate buffer (0.1M at pH 4.5). Then it was administered intraperitoneally to each animal in diabetes and D+CAP groups as a single dose of 45 mg/kg [17]. Blood samples were drawn from all rats 72 h after STZ and citrate buffer administration. Before blood samples were taken, all subjects in the study were left fasting for 8 h. Rats with blood glucose levels of 200 mg/dL or higher in the test made with a glucometer (Accu-Chek-Go, Roche) were considered diabetic [18].

Preparation and Application of CAP

CAP (Sigma, St Louis, MO, USA) was dissolved in 10% ethanol, 1% Tween 20, and 80% distilled water and administered subcutaneously to each rat in the CAP and D+CAP groups as a dose of 1 mg/kg on the 3rd day and following every day until the end of the study.

Histological Procedure

The collected kidney samples were stored in 10% formalin solution. Then the tissues were prepared by going through routine histological procedures. Slides were sectioned from the kidney tissues by using a microtome (Leica-RM2125 RTS, Germany). Crossman's triple staining, Periodic Acid Schiff (PAS), and Hematoxylin & Eosin (H&E) staining were applied to the sections taken for histological examinations. A light microscope (Olympus Bx51, Japan) was used to evaluate the slides histologically.

Immunohistochemical Procedure

Avidin-Biotin-Peroxidase complex (ABC) technique [19] was applied to examine the immunolocalization of leptin and ghrelin antibodies in kidneys. The slides were deparaffinized with xylene, rehydrated with alcohol, and washed with phosphate-buffered saline (PBS), respectively. After those processes, the slides were incubated for 10 min in 3% H_2O_2 (prepared in 0.1 M PBS) to inhibit endogenous peroxidase activity. After washing with PBS (3x5 min), the slides were kept in the microwave at 800 watts for 10 min to reveal antigens. The slides were washed with PBS (3x5 min) again, and incubated for 10 min with UV block serum (10%, Ultra V Block, Fremont CA) to prevent non-specific binding. The kidney tissues on slides were incubated with anti-leptin primary antibody (Ob Antibody (A-20): sc-482; Santa Cruz) (1:400), and anti-ghrelin primary antibody (Phoenix-H-031-31) (1:400) at room temperature for one hour. After the slides were washed with PBS, a secondary antibody (Biotinylated Goat Anti-Rabbit [Lab. Vision, 510.991.2800]) was added to the sections and kept at room temperature for 30 min. The slides were washed with PBS (3x5 min), then slides were incubated with streptavidin-horseradish peroxidase (Ultravision Detection System Large Volume Anti-Polyvalent, HRP, Thermo-scientific, UK) at room temperature for 15 min. Diaminobenzidine-hydrogen peroxidase (DAB) [20] was used to apply chromogen to the slides after the slides were washed with PBS again (3x5 min). Hematoxylin was used as a counterstain. Covered slides with entallen were examined under a light microscope (Olympus Bx51, Japan). The immunoreactivity scores of leptin and ghrelin in proximal tubules were determined according to the reaction intensity (strong=3, mild=2, weak=1, negative=0). For this aim, 20 tubules of each subject in each group were randomly selected, and the same histologist scored a total of 120 tubules in each

group. In order to understand whether it was the specific immunoreactivity, a negative control was prepared by performing all steps without adding a primary antibody.

Statistical Analysis

In our study, the number of proximal tubules samples used for Leptin and Ghrelin immunoreactivity scoring was subjected to G*Power (Universität Düsseldorf, Düsseldorf, Germany) analysis, and was confirmed with 96% reliability. Immunoreactivity scoring data were analysed statistically by using IBM SPSS Statistics Version 22 (IBM Corp., Armonk, NY, USA). Normality test (Shapiro-Wilk) and variance homogeneity test (Levene) were performed. P<0.05 was found for both tests and for both leptin and ghrelin. Since parametric assumptions were not met, Kruskal Wallis analysis of variance was performed. Kruskal Wallis test found P<0.05, then a One-Way ANOWA (Post-hoc, Tamhane) test was performed to find out which group caused the difference.

Results

Histological Results

Histologically the kidney tissues were seen as normal in the control, sham, and CAP groups. In the diabetic rats, it was observed that the glomerulus of Malpighi bodies in the cortex were scattered, and there were narrowing in the Bowman's capsules and glomerular contraction. Different cell sizes, cytoplasm disorders, cell scattering, and cell shedding were locally observed in the tubular epithelial cells of the diabetes group. Lymphocyte infiltration was also found between some tubules *(Fig. 1)*. In the D+CAP group, the structure of Malpighi bodies was similar to the control group. However, vacuolization and different cell

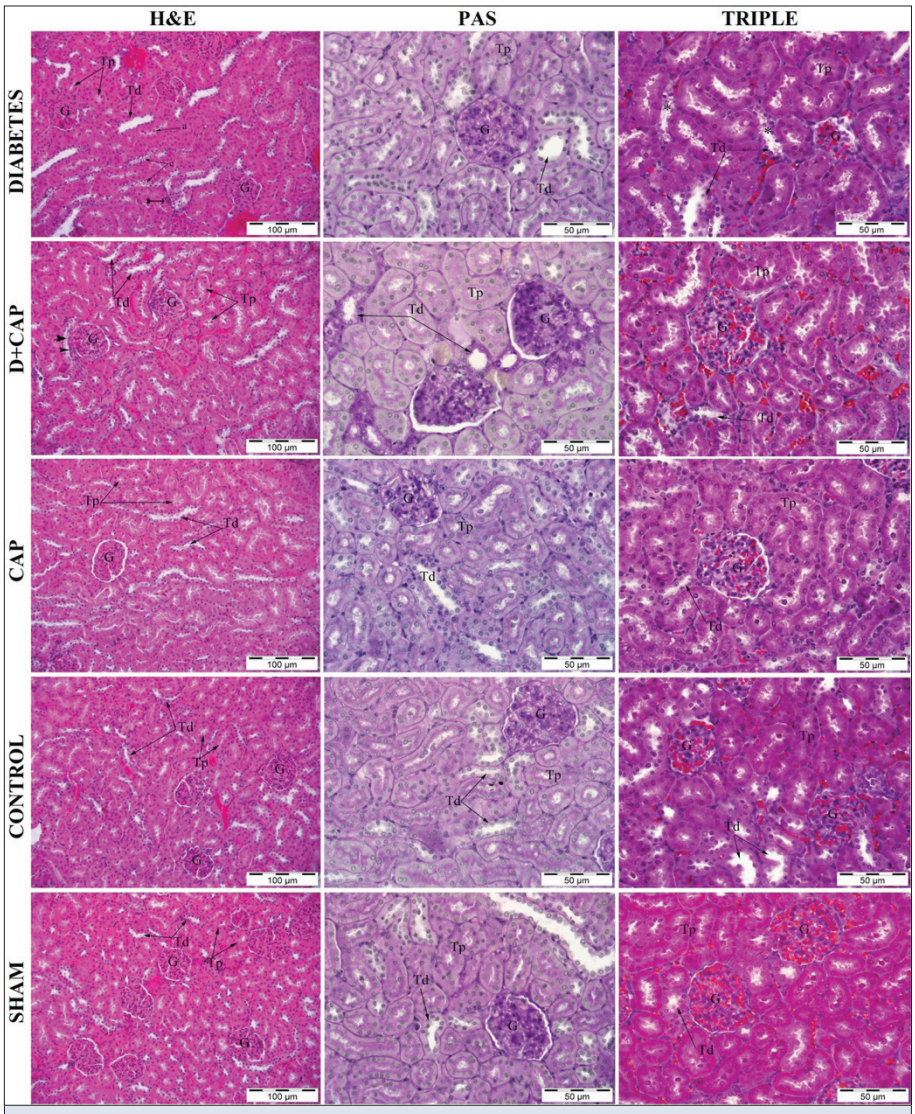

Fig 1. Histochemical staining micrographs of kidney tissues belonging to the groups. Cell irregularities *(star),* different cell size (c), cell scattering and shedding (a) in triple and H&E staining of diabetes group. Macula densa *(arrowhead),* Lympocyte cells *(tailed arrow),* Glomeruli (G), Distal tubules (Td), Proximal tubules (Tp). H&E, Scale bar: 100 µm; PAS, Scale bar: 50 µm; Triple, Scale bar: 50 µm

Table 1. *The Comparison of immunoreactivity for leptin and ghrelin in proximal tubules*

Groups	N	Number of Proximal Tubules	Immnoreactivity Score of Leptin (Mean±SD)	Immnoreactivity Score of Ghrelin (Mean±SD)
Control	6	120	1.85±0.6[a]	2.98±0.1[a]
Sham	6	120	1.87±0.6[a]	2.96±0.1[a]
CAP	6	120	1.02±0.5[b]	1.82±0.5[b]
Diabetes	6	120	2.96±0.1[c]	1.17±0.5[c]
D+CAP	6	120	1.09±0.6[b]	1.10±0.6[c]
P-value			0.001*	0.000*

[a,b,c] *Values within a row with different superscripts differ significantly at P<0.05;* ***SD:*** *Standard deviation; * Statistically significant*

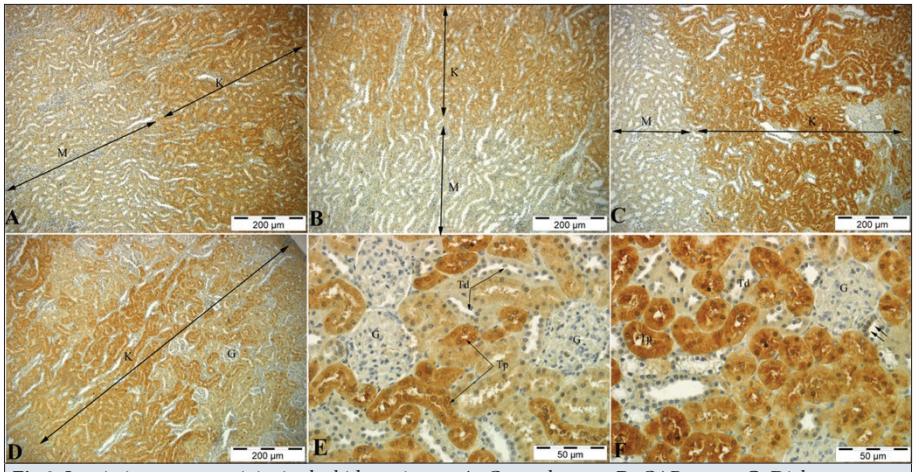

Fig 2. Leptin immunoreactivity in the kidney tissues. **A-** Control group, **B-** CAP group, **C-** Diabetes group, **D-** D+CAP group, **E-** Control group, **F-** Diabetes group. General localization of leptin immunoreactivity in groups (A, B, C, D). Glomeruli (G), Distal tubules (Td), Proximal tubules (Tp), Macula densa *(double arrows),* Nuclear Leptin immunoreactivity *(star)*

sizes were observed in the epithelial cells of the tubules in the D+CAP group *(Fig. 1)*.

In the PAS staining, we determined PAS+ areas in the basal membrane of tubules, brush border of the proximal tubules, and cytoplasm of the distal tubules in all groups. PAS positivity of glomeruli and cytoplasm of the distal tubule cells was more distinct in the D+CAP group than that of the other groups. It was also found that PAS positivity was higher in the D+CAP group and the lower in the diabetes group compared to others *(Fig. 1)*.

Immunohistochemical Results

In general, we observed that leptin immunoreactivity in the kidney was stronger in the cortex than in the medulla *(Fig. 2-A,B,C,D)*. It was seen as weaker in the distal tubule and macula densa compared to the proximal tubule. However, it was not seen in Malpighi bodies in the cortex *(Fig. 2-E,F)*. We determined leptin immunoreactivity both in cytoplasm and in nuclei but leptin showed only cytoplasmic immunoreactivity in some cells. Leptin immunoreactivity was not detected in the wall of the blood vessels.

Leptin immunoreactivity was the strongest in the diabetes group, while it exhibited the weakest immunoreactivity in the CAP and D+CAP groups, and mild immunoreactivity in the control and sham groups *(Table 1)*. In diabetes and D+CAP groups, leptin immunoreactivity was stronger in the inner cortex and weaker in the outer cortex than that of other groups in which leptin immunoreactivity had a homogeneous distribution in the cortex *(Fig. 2)*.

We observed that ghrelin immunoreactivity was widely distributed in the kidney tissue, and it was weaker in the medulla compared to the cortex. The intensity of ghrelin immunoreactivity was seen especially in the part of the cortex close to the organ capsule *(Fig. 3-A)*. Bowman capsule cells and some cells in the glomeruli had weak ghrelin immunoreactivity. Distal tubule epithelial cells had weaker immunoreactivity than that of the proximal tubule. It was noted that the ghrelin immunoreactivity in the proximal tubule epithelial cells was located and concentrated in their basal part *(Fig. 3-B,C)*. The ghrelin immunoreactivity was usually observed in cytoplasm, but in some cells, it was seen in both cytoplasm and nuclei. In addition, ghrelin immunoreactivity was determined to be widespread in the medulla, but weaker than in the cortex *(Fig. 3-D,E,F)*.

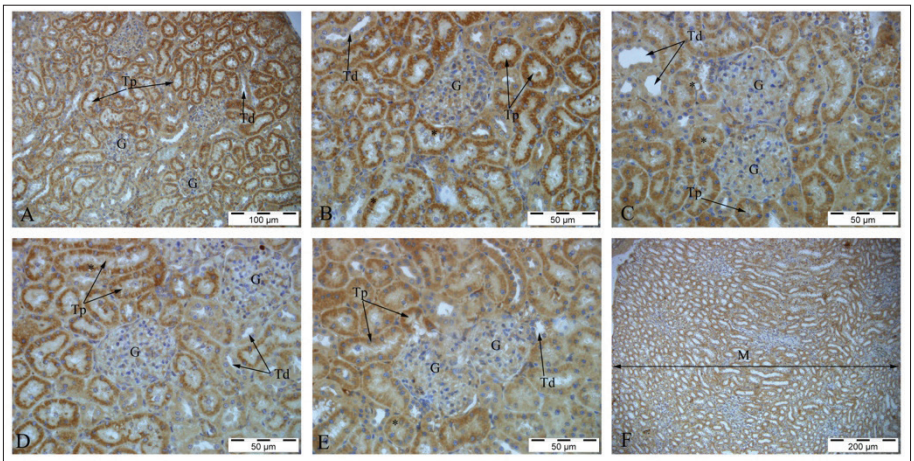

Fig 3. Ghrelin immunoreactivity in the kidney tissues. **A**- Control group, **B**- Control group, **C**- Diabetes group, **D**- CAP group, **E**- D+CAP group, **F**- Sham group. Medulla (M), Glomeruli (G), Distal tubules (Td), Proximal tubules (Tp), Nuclear ghrelin immunoreactivity *(star)*

It was found that control and sham groups had the strongest, CAP group had moderate, diabetes and D+CAP groups had the weakest ghrelin immunoreactivity when groups compared with each other (P<0.05) *(Table 1)*.

Discussion

DM is a chronic and metabolic disease characterized by the inability to produce or use insulin, and it is one of the major global health problems [21]. In our study, we aimed to examine the effects of CAP application to diabetic rats on both leptin and ghrelin immunoreactivity in the kidney, and also its effects on the kidney histological structure.

In previous studies, researchers said that STZ elevated the blood glucose level in rats at a dose of 45 mg/kg and caused DM [22,23]. STZ caused high blood glucose levels and DM by injuring the pancreas β cells [24]. In our study, STZ increased the blood-glucose level in rats at a dose of 45 mg/kg as reported in the previous studies.

Tubular atrophy, thickening of the tubular basement membrane, and interstitial inflammation may be some of the histological changes in the diabetic kidney [25]. One of these changes is the result of hyperglycaemic conditions of DM because glucose binds to the amino groups of proteins in the early phase of diabetes during hyperglycemia [26]. Under diabetic conditions, all cells of the kidney may be affected, including endothelial cells, tubule interstitial cells, podocytes, and mesangial cells. On the other hand, damage or dysfunction in any cell type can spread to all kidney cells and ultimately affect kidney function [27]. In a study, the researchers declared that the PAS stain was strong in the mesangial matrix of glomeruli in diabetic animals [28]. We observed glomerular thickening, irregularity in tubular epithelial cells, an increase in some epithelial cell size, and a narrowing as a result of thickening of the basal membranes of the tubules in the tubulointerstitial areas in diabetic kidney tissue. We also determined that the PAS reaction in the mesangial matrix of glomeruli and in the brush border of tubules was strong in the D+CAP group and the kidney histological structure of this group was similar to the control group.

Leptin and ghrelin are the main hormones by working together in opposite directions and regulating the appetite and hunger sensations [29,30]. While leptin is considered an endogenous anorexigenic hormone, ghrelin is considered the most potent endogenous orexigenic peptide, and both of them play important roles in glucose homeostasis [30,31].

A team of researchers reported that leptin immunoreactivity was observed in the distal and proximal tubules, and its immunoreactivity was found at the same location in all groups but weaker immunoreactivity in diabetic kidneys [32]. We determined intense leptin-immunoreactivity in the cortex compared to the medulla and in the proximal tubules compared to the distal tubules and macula densa. In our study, there was not any leptin immunoreactivity in the glomeruli, Bowman capsule cells, and vascular endothelial cells. In addition, unlike the previous study mentioned above, we determined more intense leptin immunoreactivity in the diabetes group than that of the others. We think that CAP decreases leptin expression when diabetes increases leptin expression because of our finding of the weakest leptin immunoreactivity in the CAP and D+CAP groups and highest leptin immunoreactivity in the diabetes group.

In a study, it was stated that ghrelin immunoreactivity was found clearly in the macula densa, which is a part of the distal tubules, and also found in the ascending loops, but not in the proximal tubules, descending loops, collecting ducts, glomeruli and interstitial cells [33]. In another study related to diabetic nephropathy, the researchers declared that ghrelin immunoreactivity was weak in the collecting

ducts, mild in the distal tubules, and negative in the glomeruli and proximal tubules. In addition, they noted that ghrelin immunoreactivity in the diabetes group was found stronger than that of the control group [34]. Unlike in the previous studies, we found ghrelin immunoreactivity in the proximal tubules and glomeruli, and even it was strong in the proximal tubules. We also determined that ghrelin immunoreactivity was located in the basal part of the proximal tubule cells. Because of this result, we consider that proximal tubules can be mistaken for distal tubules in terms of ghrelin immunoreactivity localization. In addition, we found that ghrelin immunoreactivity was weaker in the medulla compared to the cortex, and Bowman's capsule cells and some cells in the glomeruli had weak ghrelin immunoreactivity. We determined strong ghrelin immunoreactivity in the control and sham groups, mild immunoreactivity in the CAP group, and weak immunoreactivity in the diabetes and D+CAP groups. We consider that CAP has a suppression effect on ghrelin expression but diabetes has more suppression effect on its expression.

In conclusion, diabetes has bidirectional effects on appetite because it both increases leptin and decreases ghrelin. In our study, the leptin immunoreactivity increased in the diabetes group compared to the control and sham groups, while the ghrelin immunoreactivity decreased in the diabetes group compared to the control, sham, and CAP groups. We considered that the application of CAP can prevent loss of appetite by reducing the leptin expression during diabetes, and CAP cannot protect ghrelin from the adverse effects of diabetes.

Declarations

Availability of Data and Materials: The findings of the current study are available from the corresponding author (SA Bingöl) upon reasonable request.

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Conflict of Interest: The author declared that there is no conflict of interest.

Author Contributions: S.A.B, T.D, S.K.T, and S.A designed and performed the study; S.I performed immunohistochemical stain and scored immunoreactivity degree; D.G.E performed histochemical stains; S.A.B, T.D and S.I wrote the results; S.A.B. reviewed the literature and wrote the article.

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