Quercetin inhibits human sperm functions by reducing sperm $[Ca^{2+}]_i$ and tyrosine phosphorylation

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Abstract: Quercetin is widely known as potent natural antioxidant and scavenger of reactive oxygen species (ROS) and nitric oxide both in vitro and in vivo. Quercetin has a wide range of biological functions and health-promoting effects. There are more and more interests in the addition of this flavonol to various traditional food products. However, the in vitro toxicity of quercetin to mature human sperm remains unknown. In this study, we investigated the in vitro effects of quercetin on human sperm functions. The results showed that the total sperm motility were significantly inhibited compared to the controls following exposure to 100, 200 and 400μM quercetin for 6 and 12h; quercetin did not affect human sperm viability. The acrosome reaction and capacitation induced by progesterone were dose-dependently inhibited by quercetin. Furthermore, quercetin induced a significantly decrease of human sperm $[Ca^{2+}]_i$ after 2 min above 50 μM, and dose-dependently decreased the protein-tyrosine phosphorylation of human sperm. Our results indicated that quercetin may decrease sperm $[Ca^{2+}]_i$, suppresse tyrosine phosphorylation, and subsequently inhibit sperm functions.

Keywords: Quercetin, sperm, $[Ca^{2+}]_i$, tyrosine phosphorylation.

INTRODUCTION

An estimated 15% of couples globally are affected by infertility, up to 48.5 million couples, and infertility affects at least 30 million men worldwide (Agarwal *et al*., 2015). In China and some Asian countries, the seeds of *Cuscuta chinensis* (Tu-Si-Zi, TSZ) have been used for the treatment of male infertility for thousand years (Yang *et al*., 2011). Quercetin, a major component of TSZ, is widely distributed in plants and a prominent constituent of dietary supplements (Jain *et al*., 2016). Thus, quercetin has been widely used in clinical application.

Quercetin is widely known as potent natural antioxidant and scavenger of reactive oxygen species (ROS) and nitric oxide both in vitro and in vivo (Nabavi *et al*., 2012). Quercetin has a wide range of biological functions and health-promoting effects, such as anticancer, anti-inflammatory (Karuppagounder *et al.*, 2016), antibacterial activities (Nabavi *et al.*, 2015), antidiabetic (Eid *et al.*, 2015) and hepatoprotective (Ali *et al*., 2016). It was found that 248mM quercetin down regulated expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines (Davis *et al.*, 2000). The anticancer activity of quercetin has been widely studied, but in recent years, anti-obesity activity of quercetin has been more and more attention.

Calcium (Ca^{2+}) signal transduction and tyrosine phosphorylation are very important in sperm motility (Chung *et al*., 2014). Intracellular Ca^{2+} and tyrosine phosphorylation residues are absolutely required for maintenance of acrosome reaction, capacitation (Alvau *et al*., 2016; Ickowicz *et al*., 2012). The calcium from intracellular calcium storage may play an important role in anti-proliferative activities of quercetin (Cui *et al*., 2015). Quercetin also limits lipopolysaccharide (LPS)-induced inflammation via inhibition of tyrosine phosphorylation that inhibits the activation of downstream signaling pathways (Endale *et al*., 2013). However, the in vitro toxicity of quercetin to mature human sperm remains unknown.

In this study, human sperm were treated with different concentrations of quercetin *in vitro.* We focused on evaluating the toxic effects of quercetinon motility, viability, acrosome reaction and capacitation of human sperm. And we wanted to investigate the possible mechanism of its affect sperm function.

METHODS AND MATERIALS

Reagents

Progesterone, quercetin (>95%), Fluo-4 AM and pluronic F-127 were purchased from Sigma Chemical Company (St. Louis, MO). G-IVF, HAS-Solution, Sperm Grad were purchased from Vitrolife.

Sperm sample collection and treatments

Spermatozoa samples used for semen evaluation were obtained from 115 normozoospermic patients that adopted sexual abstinence for 3-5 days. All the donors signed informed consent of using their semen for our experiments. The semen samples submitted to semen examination have been approved by the internal ethical committee. All the samples were liquefied, loaded on a Sperm Grad gradient (Sperm Grad 80% and 40%) and centrifuged at 690g for 30min. The lower layer containing

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the spermatozoa was collected and washed in G-IVF twice. Then they were incubated in G-IVF (Vitrolife) plus HAS-Solution (10%) at 37°C, 5% CO_2 atmosphere.

Determination of sperm motility and viability

All samples of spermatozoa suspension in 200 ml of G-IVF (Vitrolife) plus HAS-Solution (10%) contained 20×10^6 cells and were incubated at 37°C and 5% CO_2 atmosphere. Spermatozoa motility parameters were evaluated by CASA (Hamilton Thorne IVOS II, Hamilton Thorne Ltd., *Beverly, MA, USA)* after 1h, 6h, 12h of incubation. Then the kinetic parameters of spermatozoa percentage were assessed. And human sperm were treated with different concentrations of quercetin for 1h, 6h, 12h, 37°C, 5% CO_2 incubator. Eosin-nigrosin staining was used to examine the sperm viability. The staining solution containing 0.67% Eosin-Y and 10% nigrosin was dissolved in 0.9% sodium chloride. If the sperm head stained red, it was defined as dead, while viable sperm were not stained. For each sample, a minimum of 200 spermatozoa were examined at least and categorized.

Evaluation of capacitation and the acrosome reaction

Pisumsativum agglutinin labeled by fluorescein isothiocyanate (FITC-PSA; Sigma) is used widely to assess acrosome reaction (Venkatesh *et al.*, 2011). Chlortetracycline (CTC) staining was used to evaluate the capacitation as previously described(Perry *et al*., 1995). A fluorescence microscope (Nikon, Japan) was used to view the spermatozoa smears at 450–490nm excitation. 200 spermatozoa were examined and categorized at least for each sample.

Measurement of sperm $[Ca^{2+}]_i$

The fluorescent Ca^{2+} indicator, Fluo-4 AM, was used to measure the change of human sperm $[Ca^{2+}]_i$ in 96-well plates with the Tecan Infinite 200 PRO (TECAN, Switzerland) as previously described (He *et al*., 2016). Briefly, human sperm were loaded with 5mM Fluo-4 AM and 0.05% Pluronic F-127 for 30 min at 37°C in the dark. After incubation, excess dye was removed by centrifuged at 300g for 5 min and the pellet was resuspended with G-IVF. Each well was filled with 50mL of the sperm suspension, and the fluorescence was excited at 503nm and the emission was recorded at 525nm. The fluorescence intensity was recorded before and after manual injection of 50 ml (1:1 dilution) G-IVF containing different concentrations of quercetin. The sperm $[Ca^{2+}]_i$ was calculated by the formula, $\Delta F/F_0$ (F_0, the mean fluorescent intensity before adding the chemicals; F, the fluorescent intensity recorded at every time point; $\Delta F = F-F_0$).

Western blotting

The human sperms were treated with different concentrations of quercetin at 37°C in a 5% CO_2 incubator for 3 h. According to previously published methods, the sperm proteins were isolated (He *et al*., 2016). 50μg of protein was fractionated by SDS-PAGE and transferred to polyvinylidenedifluoride (PVDF) membranes. The PVDF membranes were blocked in 5% milk–Tris-buffered saline-Tween 20 (TBST) buffer for 1 h and incubated with the anti-phosphotyrosine monoclonal antibody 4G10 (Merck Millipore, Germany) and anti-actin (Sigma, USA) at a 1:1000 dilution overnight. After that, the PVDF membranes washed with TBST and incubation with HRP-conjugated goat anti-mouse IgG (Thermo Scientific, USA). Then, membranes were exposed by the ECL detection kit (Thermo Scientific, USA).

STATISTICAL ANALYSIS

Data are expressed as mean ±SEM. Differences between the controls and the treated samples were assessed with One-way ANOVA analysis. Statistically significant differences were determined at $P<0.05$ by the statistical software SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

To evaluate the effect of quercetin on sperm motility, we incubated the sperm with different concentrations of quercetin in G-IVF plus HAS-Solution (10%), for 1h, 6h and 12h. The computer-assisted sperm analysis system (CASA) was used to test the sperm motility. The results revealed that the total sperm motility were significantly inhibited compared to the controls following exposure to 100, 200 and 400μM quercetin for 6 and 12 h; the 1h exposure did not affect human sperm mobility (fig. 1A). In this study, eosin-nigrosin staining was used to examine the sperm viability after incubation. The result showed that exposure to quercetin did not affect human sperm viability (fig. 1B), indicating quercetin has no in vitro spermicidal effect. These results suggest that human sperm motility inhibited by quercetin was not a result of decreased sperm viability.

It is well known that capacitation and the acrosome reaction (AR) of sperm are very important for fertilization under natural conditions. They are essential for sperm to penetrate the oocyte. Thus, we evaluate the effects of quercetin on capacitation and AR in human sperm. The results showed that human sperm the spontaneous acrosome reaction and capacitation were not affected by the concentrations of quercetin used in this study (fig. 2A and C). On the contrary, the acrosome reaction and capacitation induced by progesterone, a very important physiological process for fertilization, were dose-dependently inhibited by 100μM quercetin (fig. 2B and D). These results indicated that quercetin above 100 μM may disturb the progesterone regulated signal pathways in sperm fertility.

Our results showed that quercetin inhibited human sperm motility and progesterone induced acrosome reaction and capacitation. It was known that intracellular Ca^{2+}

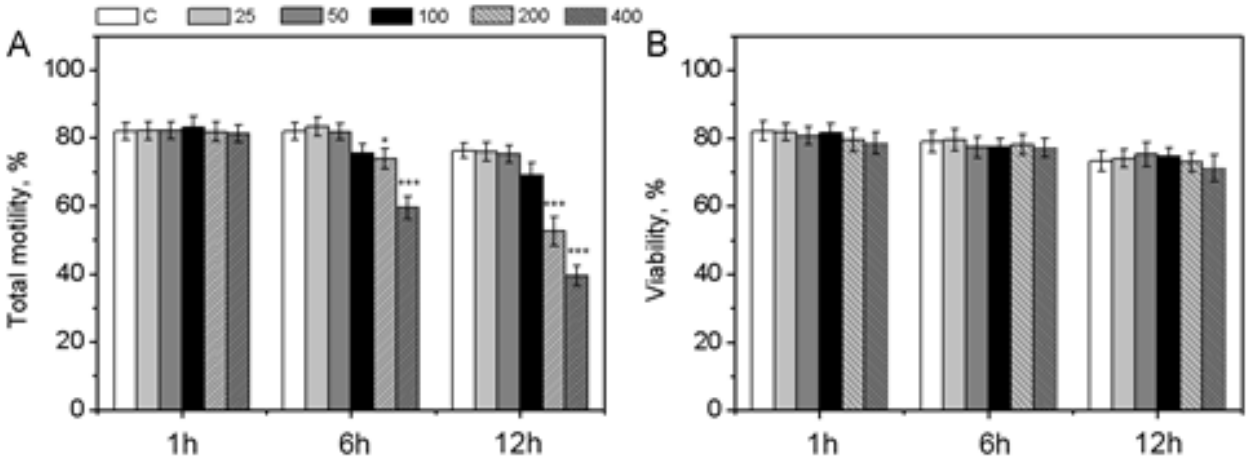

Fig. 1: The effect of quercetin on sperm motility and viability in vitro. (A) Human sperm were incubated G-IVF (Vitrolife) plus HAS-Solution (10%) with different doses of quercetin at 37°C and 5% CO_2 incubator for 1, 6 and 12 h, respectively. The total motility was analyzed by Computer-Assisted Sperm Analysis (CASA). (B) The incubated sperms were examined by eosin-nigrosin stain. A minimum of 200 sperms were counted for each assay. Bar: mean ±SEM. *p<0.05, **p<0.001 and ***p<0.0001, t-test, n=10.

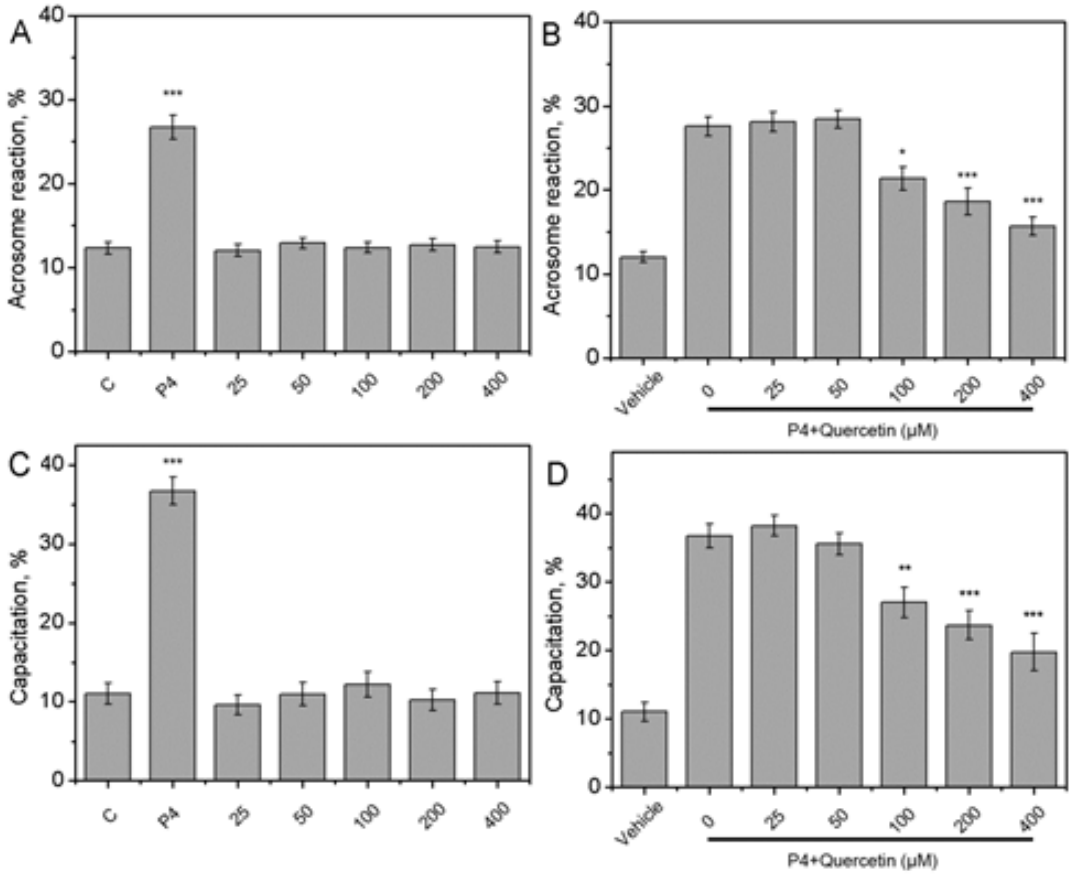

Fig. 2: The effect of different concentrations of quercetin on acrosome reaction and capacitation of human sperm in vitro. To assess the effect of quercetin on sperm acrosome reaction (A and B), human sperm were treated with quercetin (0-400μM) at 37°C, 5% CO_2 incubator for 3h (A). For examining the effect of quercetinon progesterone (P4)-induced acrosome reaction, vehicle (0.1% DMSO, a negative control) and 10mM P4 plus 0-400μMquercetinwere added and incubated for 3h (B). A total of 200 sperm were counted in each assay to evaluate PSA-FITC staining. To assess the effect of quercetin on sperm capacitation (C and D), human sperm were treated as described above. A total of 200 sperm were counted in each assay by CTC stain. Bar: mean ±SEM. *P<0.05, **P<0.01 and ***P<0.001, One-way ANOVA analysis, n=10.

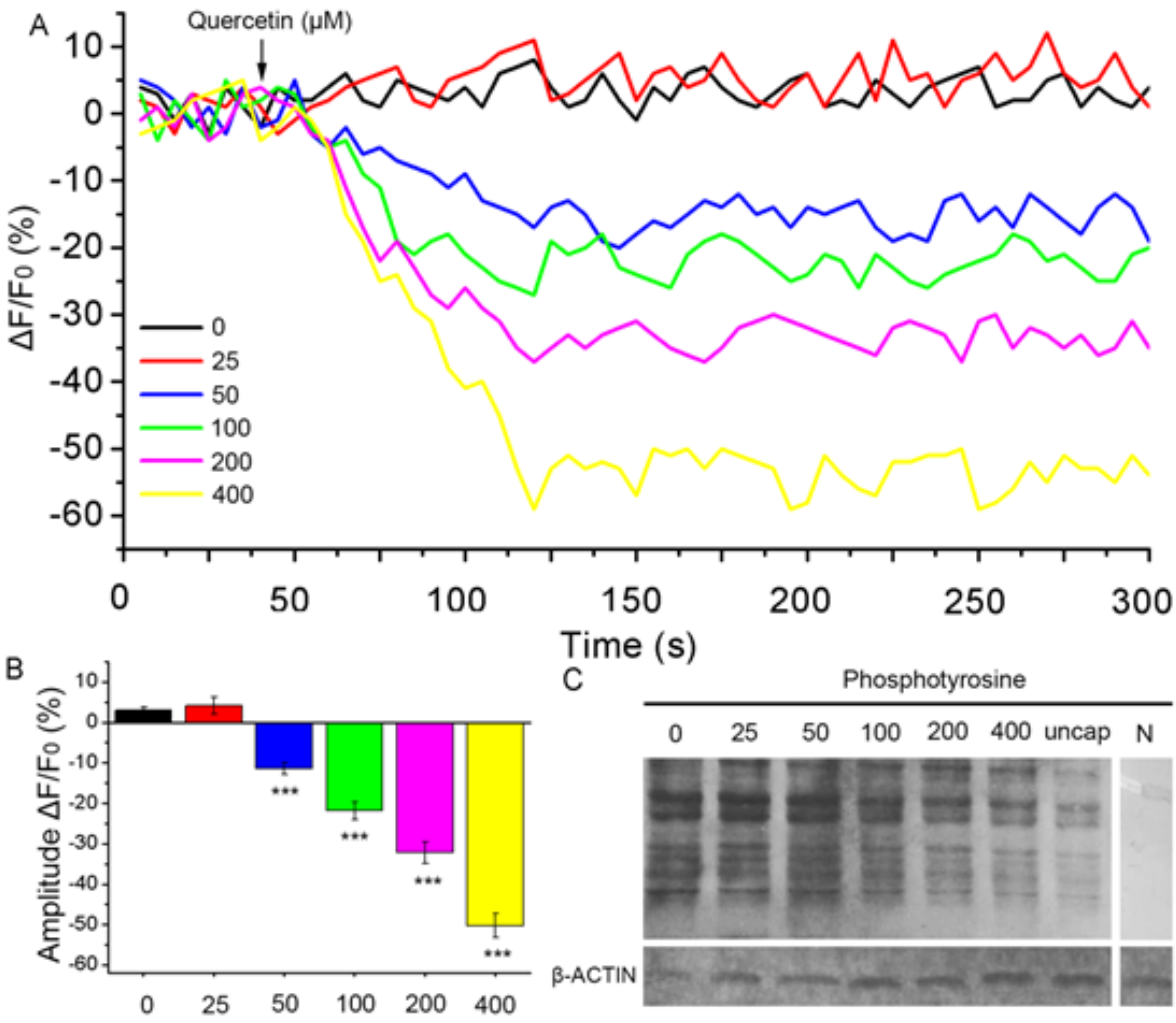

Fig. 3: The effect of different concentrations of quercetin on human sperm $[Ca^{2+}]_i$ and tyrosine phosphorylation. The human sperm $[Ca^{2+}]_i$ was monitored by 5 mMFluo4-AM described in Materials and Methods. A time-course curve showed the real-time changes of sperm $[Ca^{2+}]_i$ (A). Arrows indicated the time of quercetin (0-400μM) added into sperm samples. The inhibition effects of quercetin on human sperm $[Ca^{2+}]_i$ was calculated as the amplitude $\Delta F/F_0$ from time-course curve by statistical analysis (B). Bar: mean ±SEM. *P<0.05, **P<0.01 and ***P<0.001, One-way ANOVA analysis, n=10. (C) Sperm proteins isolated from the human sperm capacitated G-IVF plus HAS-Solution (10%) containing different concentrations of quercetin (0-400μM) or incubated inG-IVF (Uncap) without HAS-Solution (10%), respectively. The tyrosine phosphorylation was measured by western blot using anti-phosphotyrosine monoclonal antibody 4G10 (Merck Millipore, Germany) and the preimmune IgG (negative control, N).

concentration ($[Ca^{2+}]_i$)-dependent processes played an important role in these functions of human sperm. Thus, We examined the effects of quercetin on the $[Ca^{2+}]_i$ in human sperm. The results indicated that quercetin induced a significantly decrease of human sperm $[Ca^{2+}]_i$ after 2 min above 50μM (fig. 3A and B). Meanwhile, the sperm $[Ca^{2+}]_i$ were not affected by the 25 μM quercetin (fig. 3). These results imply that quercetin inhibits the sperm functions may be due to decreasing the sperm $[Ca^{2+}]_i$ in vitro. In human sperm, it is also regarded protein tyrosine-phosphorylation as an essential process for sperm functions (motility, the acrosome reaction and capacitation). So we examined the tyrosine-tyrosine phosphorylation of the human sperms exposed to different concentrations of quercetin G-IVF plus HAS-Solution (10%) at 37°C in a 5% CO_2 incubatorfor 3h. The results showed that quercetin (100-400μM) dose-dependently decreased the protein-tyrosine phosphorylation of human sperm. These results indicated that the inhibited effects of quercetinon AR and capacitation may relate the inactivating tyrosine kinase in human sperm.

DISCUSSION

Quercetin is the major bioflavonoid in the human diet and in many dietary supplements. A lot of studies were focused on the benefit for health of quercetin (Jain *et al*., 2016), but few studies were involved in reproductive

toxicity of quercetin. In this study, we first evaluated the in vitro toxic effects of quercetin on human sperm functions including viability, motility, acrosome reaction and capacitation.

The anti-oxidant, cardioprotective properties, anti-carcinogenic, and anti-inflammatory are several key biological functions ascribed to quercetin (Ajibade *et al*., 2016; Shanely *et al*., 2010). Because the beneficial effects of quercetin are well known by people, there are more and more interests in the addition of this flavonol to various traditional food products (Harwood *et al.*, 2007). But quercetin exposure caused DNA single strand breaks, chromosomal aberrations and micronucleus formation was found in many kind cells in vitro (Cantero *et al.*, 2006; Leal *et al.*, 2003; Rahman *et al.*, 1989). It has been proved that it have mutagenicity/genotoxicity in vitro, but has not been confirmed by in vivo experiments (Aguirre *et al*., 2011; Utesch *et al*., 2008). In our study, 200μM quercetin suppressed the motility of sperm in vitro in 6h incubation (fig. 1). The progesterone-induced acrosome reaction and capacitation of human sperm in vitro were also inhibited by 100μM quercetin (fig. 2). These results indicated that quercetin inhibits some of human sperm functions. Rats could reach a serum concentration of 133 mM quercetin, mainly in sulfated and glucuronidated forms, after eating a diet supplemented with 0.2-percent quercetin for three weeks (Davis *et al*., 2000). The diet by consumers with a high fruit and vegetable intake (i.e., 200-500mg/day) did not induce toxicologically significant adverse effects, proved by long-term, dose level, animal studies (Harwood *et al*., 2007).Thus, although regular daily quercetin intake may not induce reproductive toxicity, utilizing high doses of quercetin in clinical applications would be paid more attention. The reproductive toxicity of quercetin should be further studied in future.

The intracellular Ca^{2+}concentration plays essential roles in the modulation of sperm functions, such as motility, acrosome reaction and capacitation (Jaldety and Breitbart, 2015; Luo *et al*., 2015a; Luo *et al.*, 2015b; Publicover *et al.*, 2008). Additionally, the reduction of protein-tyrosine phosphorylation is absolutely required for the decrement of sperm motility, capacitation and acrosome reaction (Alvau *et al*., 2016; Stival *et al*., 2016). A lot of studies proved that tyrosine phosphorylation is a highly Ca^{2+}-dependent process (Chung et al., 2014; Tamburrino et al., 2014). Our results are consistent with the idea that quercetin decreases sperm $[Ca^{2+}]_I$ (fig. 3), suppresses tyrosine phosphorylation (fig.3) and subsequently inhibits sperm functions.

In conclusion, quercetin may inhibit human sperm motility, acrosome reaction and capacitation via decreasing sperm $[Ca^{2+}]_i$ and suppressing tyrosine phosphorylation. It would be paid more attention in utilizing high doses of quercetin in clinical applications.

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