Gold Nanoparticles Improve Metabolic Profile of Mice Fed a High-Fat Diet

Hui Chen [1,2]*, Jane PM Ng [1]*, Yi Tan [1], Kristine McGrath [1,2], David P Bishop [3], Brian Oliver [1,2], Yik Lung Chan [1], Michael B Cortie [4], Bruce K Milthorpe [1,2], Stella M Valenzuela [1,2#]

1. Molecular Biosciences Team, School of Life Sciences, Faculty of Science, University of Technology Sydney. NSW 2007, Australia
2. Centre for Health Technology, University of Technology Sydney. NSW 2007, Australia
3. School of Mathematical and Physical Sciences, Faculty of Science, University of Technology Sydney, NSW, 2007, Australia
4. Institute for Nanoscale Technology, University of Technology Sydney. NSW 2007, Australia

* These authors contributed equally to this work.

#Corresponding Author

Associate Professor Stella M Valenzuela

Molecular Biosciences Team, School of Life Sciences, Faculty of Science, University of Technology Sydney. NSW 2007, Australia.

Phone: 61 2 9514 1917. Fax: 61 2 9514 8206. E-mail: Stella.Valenzuela@uts.edu.au

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Abstract

Background: Obesity is a high risk for multiple metabolic disorders due to excessive influx of energy, glucose and lipid, often from a western based diet. Low-grade inflammation plays a key role in the progression of such metabolic disorders. The anti-inflammatory property of bulk gold has been used in treating rheumatoid arthritis in the clinic, not its form at nanoscale. Previously we found that pure gold nanoparticles (AuNPs, 21nm) also possess anti-inflammatory effects on the retroperitoneal fat tissue following intraperitoneal injection, by downregulating tumor necrosis factor (TNF) α. However, whether such an effect can change the risk of metabolic disorders in the obese has not been well studied.

The study employed C57BL/6 mice fed a pellet high fat diet (HFD, 43% as fat) that were treated daily with AuNPs [low (HFD-LAu) or high (HFD-HAu) dose] via intraperitoneal injection for 9 weeks. In the *in vitro* study, RAW264.7 macrophages and 3T3-L1 adipocytes were cultured with low and high concentrations of AuNPs alone or together.

Results: The HFD-fed mice showed a significant increase in fat mass, glucose intolerance, dyslipidemia, and liver steatosis. The HFD-LAu group showed an 8% reduction in body weight, ameliorated hyperlipidemia, and normal glucose tolerance; while the HFD-HAu group had a 5% reduction in body weight with significant improvement in their glucose intolerance and hyperlipidemia. The underlying mechanism may be attributed to a reduction in adipose and hepatic local proinflammatory cytokine production, eg.TNFα. *In vitro* studies of co-cultured murine RAW264.7 macrophage and 3T3-L1 adipocytes supported this proposed mechanism.

Conclusion: AuNPs demonstrate a promising profile for potential management of obesity related glucose and lipid disorders and are useful as a research tool for the study of biological mechanisms.

Background

Obesity is an important risk factor for multiple metabolic disorders, including glucose intolerance and hyperlipidemia. The current global surge in obesity has seen a staggering 800% increase in demand for weight-loss surgical procedures over the last decade, as a means of controlling these metabolic disorders (1) . This increase is also driven by the disappointingly low success rate of weight-loss medications and interventions, as well as the difficulties faced by individuals trying to maintain ideal body weight following initial weight loss. For example, in a recent trial, the latest approved injectable weight loss medication, Liraglutide (Saxenda) has been shown to induce ~6% of total body weight loss after 56 weeks of treatment (2). However, this weight loss effect required daily adherence to a strict low-caloric diet and ongoing support by dieticians, making its implementation difficult to achieve outside of a closely controlled environment (2). Therefore, there still remains an urgent and growing need for effective strategies to deal with the global obesity pandemic. Herein, we present intriguing evidence that gold nanoparticles (AuNPs) may serve as a novel therapeutic agent in the treatment and control of obesity and its related blood glucose and lipid disorders.

There is already historical precedence for the use and application of bulk gold and gold salts within clinical practice (3). It is now becoming evident that AuNPs share similar therapeutic potentials (4). Nanomaterials have been widely applied in medicine as biochemical sensors, contrast agents in imaging, and drug delivery vehicles revolutionizing current disease treatment and diagnosis (4). However, the function and toxicity of AuNPs differ subtantially depending on the size and shape with AuNPs larger than 15 nm comparatively nontoxic (5).

Previously, we injected unmodified spherical AuNPs of 21 nm diameter into chow-fed lean mice (6). The AuNPs accumulated rapidly in the abdominal fat tissue after a single intraperitoneal (IP) injection. AuNP-treated mice showed significant reduction in abdominal fat mass compared to non-treated control mice, along with reduced mRNA expression of the pro-inflammatory cytokines, tumor

necrosis factor (TNF)-α, in the abdominal fat tissue (6). This is of great interest, as TNF-α has been frequently linked to the comorbidities related to obesity (7). In chronic obesity, excess triglyceride storage in the fat tissue can up-regulate adipose triglyceride lipase (ATGL) to increase basal lipolysis (8). Consequently, adipose tissue macrophage (ATM) infiltration and accumulation into the fat tissue is also increased, which promotes inflammatory responses in the adipose tissue by directly engaging toll-like receptors (TLR) to induce production of cytokines, such as TNFα (9). For these reasons, TNFα expression is positively correlated with body mass index, hyperlipidemia, insulin resistance, and glucose intolerance (10, 11). Either reducing ATM recruitment or inhibiting ATM cytokine release can lead to fat loss and improved insulin sensitivity in obese mice (9, 12). This highlights the essential roles of ATM-related cytokines in the development of metabolic disorders in obesity. The down-regulation of pro-inflammatory cytokines in our previous study was linked to reduced ATM activity, rather than reduced cell number (6). In addition, the abdominal fat loss induced AuNP treatment was also of interest for its potential to treat obesity.

Although the anti-inflammatory property of bulk gold and AuNPs has been clinically used for treating rheumatoid arthritis (3), the injectable AuNP preparation has not been reported for managing adiposity and metabolic disorders in obesity. Therefore, in the current study we IP injected AuNPs into mice fed a high-fat diet (HFD) for 9 weeks to examine the effect on fat accumulation and obesity related metabolic disorders. In addition, our *in vitro* studies investigated the direct impact of the AuNPs on adipocyte and macrophage interactions. The knowledge gained from this study will serve to inspire new, original and more effective therapeutic approaches that involve direct targeting of intracellular pathways in adipocytes and/or macrophage cells.

Methods:

Animal experiments:

Male C57Bl/6 mice (8 weeks, Animal Resource Centre, WA, Australia) were then randomly divided into 4 groups (*n*=20, Table 1). Control group (Chow-C) were fed chow (Gordon's Specialty Stockfeeds, NSW, Australia) and injected with vehicle; HFD group (HFD-C) was fed a HFD (20 kJ/g, 43% fat, Cat. SF03-020, Specialty Feeds, WA, Australia) *ad libitum* and injected with vehicle; low dose AuNP (HFD-LAu) group fed a HFD and received AuNP (0.785µg Au/g, IP); and high dose AuNP (HFD-HAu) group fed a HFD and received AuNP (7.85µg Au/g, IP) determined according to our previous study (6). The HFD has been repeatedly used to induce obesity in rodents by us (13-18). The chow-fed mice treated with AuNP was not adopted in this study as we have shown the fat loss effect in lean mice (6) and lean humans rarely requires weight loss treatment. AuNPs were prepared as previously described (6), and injection was performed at 10 am daily for nine weeks. Food intake and body weight was monitored weekly. IP glucose tolerance test (IPGTT) was performed at 8 weeks in randomly selected mice from each group as previously described (15). The area under the curve (AUC) of glucose levels was calculated for each mouse. Tissues were harvested at 9 weeks after Pentothal (0.1 mg/g, IP, Abbott Diagnostics, NSW, Australia) anesthesia. Blood glucose was measured (Accu-Check®, Roche, CA, USA) and plasma was stored at -80°C. Heart, spleen, kidneys, liver, and abdominal fat pads were weighed and either fixed in 10% formalin or snap frozen in liquid nitrogen and stored at -80°C. All tissue analysis was performed in a blind manner and the results were only identified before data analysis.

In vitro experiments see supplementary materials

Biochemical analysis:

Plasma and cell supernatant triglycerides were measured using an in house assay using glycerol standards (Sigma-Aldrich, MO, USA) and triglyceride reagent (Roche Diagnostics, NJ, USA). Nonesterified free fatty acid (NEFA) was measured using a NEFA kit (WAKO, Osaka, Japan) (19).

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using commercial kits (Dialab Ltd., Vienna, Austria) as an indicator of liver cell damage. Plasma cholesterol concentration was measured using the Cholesterol CHOD-PAP with ATCS kit (Dialab Ltd., Vienna, Austria).

Quantitative real-time PCR:

Total RNA was isolated (*n*=5-10 randomly selected from each group, cells *n*=8-10) using TRI reagent (Sigma-Aldrich, MO, USA). First-strand cDNA was synthesised using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant Kit (Promega, WI, USA) (20, 21). Pre-optimized TaqMan® probe/primers (Supplementary Tables S1, Life Technologies, CA, USA) and SYBR® Green premiers (Supplementary Table S2, Bio-Rad, CA, USA) (22) were used for the real-time PCR (Eppendorf Realplex^2, Hamburg, Germany). The genes of interest were normalized against the housekeeping gene 18s rRNA (Table S1). The average value of the control was assigned as the calibrator, against which all other samples are expressed as a fold difference.

Immunohistochemistry:

Formalin fixed liver and abdominal fat samples (n=5) were embedded in paraffin and sectioned (4 μm). To explore F4/80 posative cells sections were incubated with a rabbit anti-mouse F4/80 (Abcam, Cambridge, UK) primary antibodies, and visualised using the horseradish peroxidase anti-rabbit Envision system (Dako Cytochemistry, Tokyo, Japan). The sections were then counterstained with haematoxylin. Three images from each section were captured and used for analysis. The F4/80-expressing cells were counted and expressed as the percentage of total cell number for a sample total number of nuclei and the number of nuclei of for each field.

Statistical analysis:

The results were expressed as mean ± standard error of the mean (S.E.M). The data was analyzed using one-way ANOVA, followed by *post hoc* Bonferroni tests (Statistica 10. StatSoft Inc. OK, USA), if normally distributed. If the data was not normally distributed, they were log transformed to achieve normality of distribution before they were analyzed. The glucose levels during IPGTT were analyzed using one-way ANOVA with repeat measures followed by *post hoc* Bonferroni test. $P<0.05$ was considered significant.

Results

1. *In vivo* Animal Study

1.1. Anthropometry

Four groups of mice started with similar body weight (Table 1). At 9 weeks post-treatment, the HFD-fed (HFD-C) was 35% heavier than the Control (Chow-C) group, with significantly increased organ and fat masses, as well as blood lipid cholesterol and NEFA concentrations ($P<0.05$, Table 1). Adipocyte size was more than doubled in the HFD-C group ($P<0.01$ *vs.* Chow-C, Supplementary Figure S1). Plasma ALT and AST levels were ~3 and 5 times higher in the HFD-C group ($P<0.05$ *vs.* Chow-C, Table 1). Blood glucose levels during IPGTT were also significantly higher in the HFD-C group than the Chow-C, from 15–90 min post glucose injection ($P<0.05$, Figure 1a), with 60% greater AUC value ($P< 0.05$, Figure 1b).

The two groups of mice treated with AuNPs consumed more energy than the Chow-C and HFD-C groups ($P<0.05$, Table 1). However, the body weights of the HFD-Lau and HFD-HAu groups were 8% and 5% smaller than the HFD-C mice, respectively ($P<0.05$). Smaller fat masses were observed in the AuNP-treated mice ($P<0.05$ retroperitoneal, HFD-C *vs.* HFD-HAu; mesenteric, HFD-C *vs.* HFD-LAu and HFD-HAu, Table 1). However, the fat cell size was larger in the HFD-LAu group, but smaller in the HFD-HAu group (both $P<0.01$ *vs.* HFD-C, Figure S1). Both AuNP-treated groups had significantly lower blood lipid levels than the HFD-C group ($P<0.05$) with nearly normalized liver

AST and ALT levels (P<0.05, Table 1). These results suggest a lipid lowering effect by the AuNPs and long-term safety and benefit to the liver. During IPGTT (Figure 1a), the HFD-LAu group did not develop glucose intolerance; while the HFD-HAu group had significantly improved glucose clearance at 60–90 min (P<0.05 *vs.* HFD-C, Figure 1a). AUC showed similar changes as the blood glucose levels in all three HFD groups (Figure 1b).

1.2 Organ distribution of the AuNPs

After 9 weeks, trace amounts of gold where detected in the Chow-C and HFD-C mice (Supplementary Table S3) by inductively-coupled plasma-mass spectrometry (Supplementary material), which has also been observed in humans (3). In both the HFD-LAu and HFD-HAu groups, the highest concentration of gold was found in the abdominal fat tissue, followed by the spleen and the liver (P<0.05 *vs.* Chow-C and HFD-C, Supplementary Table S3). In the HFD-LAu group, gold was negligible in the kidney, brain and heart (Supplementary Table S3). In the HFD-HAu group, gold was still detectible in the kidney and brain, but not the heart (P<0.05 *vs.* Chow-C, HFD-C and HFD-LAu groups, Supplementary Table S3).

1.3 mRNA expression of inflammatory and metabolic markers, and the percentage of macrophages in the fat and liver

In the retroperitoneal fat, TNFα and TLR-4 mRNA levels were significantly up-regulated following long-term HFD consumption (P<0.05 *vs.* Chow-C, Figure 2a, b). On the other hand, serum amyloid A (SAA)-1 level was more than 5 times that of the control mice, however without statistical significance (Figure 2c). However, the percentage of macrophages was not changed by HFD consumption (Figure 2d). In the HFD-LAu group, TNFα and SAA-1 mRNA expression levels were significantly down-regulated (P<0.05 *vs.* HFD-C, Figure 2a,c); as was TLR-4 level by ~50% however without statistical significance (Figure 2b). In the HFD-HAu group, both TLR-4 and SAA-1 expression levels were significantly reduced (P<0.05 *vs.* HFD-C, Figure 2b,c). The percentage of

macrophages was halved in HFD-LAu group although without statistical significance, which was not altered in HFD-HAu group (Figure 2d).

In the liver, HFD consumption alone significantly up-regulated TNFα mRNA expression ($P<0.05$ *vs.* Chow-C, Figure 2e). SAA-1 mRNA levels were nearly doubled in the HFD-C group however without statistical significance (Figure 2g). The percentage of macrophages was significantly increased by HFD consumption ($P<0.05$ HFD-C *vs.* Chow-C, Figure 2h). Both TNFα and TLR-4 mRNA expression levels were significantly reduced by HFD-LAu treatment; however TLR-4 and SAA-1 expression levels were increased in HFD-HAu group ($P<0.05$ *vs.* HFD-C, Figure 2). AuNP-treatment normalized the percentage of macrophages relative to control animals ($P<0.05$ *vs.* HFD-C, Figure 2h).

In the fat tissue, mRNA levels of glucose transporter (GLUT)4 and adiponectin were significantly reduced; while ATGL, carnitine palmitoyl transferase (CPT-1α), and leptin were significantly increased following HFD consumption ($P<0.05$ *vs.* Chow-C, Figure 3a,b,c,g). Conversely, HFD-LAu treatment significantly lowered CPT-1α mRNA expression ($P<0.05$ *vs.* HFD-C, Figure 3c); while HFD-HAu treatment significantly down-regulated leptin, but increased adiponectin mRNA expression ($P<0.05$ *vs.* HFD-C, Figure 3e,g).

In the liver, GLUT4 and Sterol regulatory element-binding transcription factor (SREBP)-1c mRNA expression levels were significantly up-regulated; while CPT-1α mRNA expression was significantly down-regulated following HFD consumption ($P<0.05$ *vs.* Chow-C, Figure 4a,c,d). Although fatty acid synthase (FASN) was increased by 35% and forkhead box O1 (FOXO1) expression was up-regulated by more than 50%, neither was significant (Figure 4e,f). HFD-LAu group had significantly reduced SREBP-1c and FASN mRNA expression ($P<0.05$ *vs.* HFD-C, Figure 4d,e). The HFD-HAu group had significantly increased GLUT4 ($P<0.05$ *vs.* HFD-C, Figure 4a), and higher levels of FOXO1 compared to the Chow-C group ($P<0.05$, Figure 4f).

2 *In vitro* studies

2.1 Effects of AuNPs on MΦ cell lines

Low concentration of AuNPs reduced cell viability at 24h and 72h post incubation ($P<0.05$ *vs.* MΦ-C, Supplementary Figure S2b,c). Cell viability was reduced in the MΦ-HAu group across all three time points ($P<0.05$, 0.01 *vs.* MΦ-C, Supplementary Figure S2a–c). Reactive oxygen species (ROS) levels were only significantly increased in the MΦ–HAu group at 24h ($P<0.01$ *vs.* MΦ-C, Supplementary Figure S2e).

TNFα mRNA expression was only significantly reduced in the MΦ-HAu group at 1h ($P<0.05$ *vs.* MΦ-C, Supplementary Figure S3a), but was significantly increased in both MΦ-LAu and MΦ-HAu groups at 24h ($P<0.05$ *vs.* MΦ-C, Supplementary Figure S3b). TLR-4 mRNA expression was significantly reduced in the MΦ-HAu group at both 1h and 72h ($P<0.05$ *vs.* MΦ-C, Supplementary Figure S3d,f). However, TLR-4 and TNFα protein levels were not changed by AuNPs, which were significantly increased in the positive control LPS incubated cells ($P<0.05$ *vs.* MΦ-C, Supplementary Figure S4a–c). However, AuNPs cannot suppress LPS induced increase in TLR-4 and TNFα protein levels (data not shown).

2.2 Effects of AuNPs on 3T3-L1 adipocytes

Cell viability of the mature 3T3-L1 adipocytes (Supplementary Figure S5a–c), and 3T3-L1 differentiation from fibroblast (data not shown) were not affected by AuNPs. ROS production was increased in the AD-HAu group at 24h ($P<0.05$ *vs.* AD-C, Supplementary Figure S5e). Lipid accumulated was significantly reduced in the AD-HAu group at 1h ($P<0.05$ *vs.* AD-C, Supplementary Figure S5g); it was significantly increased by 9% in this group at 72h ($P<0.01$ *vs.* AD-C, Supplementary Figure S5i). In addition, adipocyte cell size was increased in the AD-LAu group at 24h, however it was reduced in the AD-HAu group at 72 h ($P<0.05$ *vs.* AD-C, Supplementary Table

S4). Triglycerides levels secreted into the culture media were similar between the three groups at all time points (Supplementary Table S4).

GLUT-4 mRNA levels were significantly down-regulated in both AD-LAu and AD-HAu at 72h (P<0.01,0.05 *vs.* AD-C, Supplementary Figure S6c). ATGL was significantly reduced in the AD-LAu group at 24h (P<0.05 *vs.* AD-C, Supplementary Figure **S**6e). Under low ambient glucose concentration, glucose uptake was significantly reduced in the AD-HAu group at 60min (P<0.05 *vs.* AD-C, Supplementary Figure S7a); whereas under high ambient glucose concentration, glucose uptake was significantly increased at 5min in the AD-LAu group (P<0.05 *vs.* AD-C, Supplementary Figure S7b).

2.3 Effects of AuNPs on adipocytes and macrophages in co-culture (MΦ+AD)

In this co-culture system, cell viability and ROS production were similar among the groups at all three time points (Supplementary Figure S8). TLR-4 was significantly increased at 24h in the (MΦ+AD)-HAu group (P<0.05 *vs.* (MΦ+AD)-C, Supplementary Figure S9e). For the metabolic markers, at 24 h GLUT-4 and ATGL mRNA was significantly up-regulated in both (MΦ+AD)-LAu and (MΦ+AD)-HAu groups (P<0.05 *vs.* (MΦ+AD)-C, Supplementary Figure 9b,e). CPT-1α mRNA levels were up-regulated 1.3-fold in the (MΦ+AD)-HAu group versus the control group at 24 h (P<0.05 *vs.* (MΦ+AD)-C, Supplementary Figure 9h).

Discussion

In HFD-fed mice, AuNPs slowed down the development of obesity with significantly improved lipid metabolic profile. It also provided a marked protective effect against the development of glucose intolerance, which is recognized as a first step towards type 2 diabetes. In particular, the lower dose provided better outcomes. A reduction in local inflammation within the adipose tissue and the liver may service as the underlying mechanism; while the *in vitro* co-culturing data support AuNP's

287 regulation of cellular interactions between macrophages and adipocytes as orchestrating these anti-
288 inflammatory events.
289
290 In the current study, the males are not affected by periodical changes in sex hormones and are
291 therefore used for this study to prove the concept. The mice fed a HFD *ad libitum* for 9 weeks showed
292 a significant increase in their fat mass and developed glucose intolerance, dyslipidemia, and liver
293 steatosis, which are consistent with our previous studies (15, 21, 23). Liver enzyme levels were also
294 increased by several folds in the HFD-fed mice, suggesting liver cell damage. However, daily AuNP
295 injection significantly ameliorated such effects by HFD consumption, with significant improvement
296 in glucose and lipid metabolism. Liver enzyme changes may suggest a liver protection of AuNPs
297 against dietary lipid influx induced liver damage.
298
299 Clinical research suggests that loss of as little as 5% of total body weight can reduce the risk of
300 developing type 2 diabetes by 58% (24). This benefit was well supported by the current study. The
301 HFD-LAu group showed 8% less body weight and demonstrated normal glucose clearance during
302 IPGTT, while the HFD-HAu group, with 5% less body weight, demonstrated significantly improved
303 glycaemic control. It needs to be noted that this effect was achieved under the condition of free access
304 to HFD without any restriction that employed by the human clinical trial (2). Their daily caloric intake
305 was even higher than non-treated mice consuming HFD. This may be an adaptation to their reduced
306 fat mass; where smaller fat mass may be due to increased CPT-1α expression to increase fatty acid
307 oxidation for energy synthesis. Therefore, it can be postulated that combining the AuNP treatment
308 with restricted energy intake to the level of the Chow-C group may exert more pronounced weight
309 loss effect. This is yet to be confirmed by future studies. The low concentration of AuNP seems to
310 exert a better effect than the high contraction. This may be due to the aggregative nature of the AuNPs
311 at high concentration, which results in less free monodispersed AuNPs entering the tissue and the
312 circulation, as well as impacting on the cells. The effects of AuNP are well known to be highly

dependent on particle size (25). As this was the first study to show the anti-obesity effect of the AuNPs, ip injection was chosen as it is the most convenient method of AuNP delivery. In future studies, we will test the efficacy of subcutaneous injection and oral delivery, which are the common administration method in humans. In addition, for unknown reasons, the lower dose AuNPs seems to stimulate insulin secretion, which may have contributed to normalized glycaemic control in this group. This result warrants further investigation of the interaction between AuNPs and β-cells.

Increased macrophage infiltration has been suggested to contribute to the low-grade inflammation state commonly associated with obesity (26). During HFD consumption, excessive fat accumulation in the abdominal fat tissue increases the recruitment of ATMs (27), producing pro-inflammatory cytokines (e.g. TNFα), which in turn drives obesity-related metabolic disorders (28, 29), (27, 30, 31). TNFα is known to reduce free fatty acid transporter and extracellular lipoprotein lipase activity, thereafter inhibit the uptake of fatty acids into fat cells, leading to hyperlipidemia and ectopic lipid storage (eg. in the liver); while local lipid accumulation is a key contributor to insulin resistance (32). TNFα itself can also interrupt insulin signaling, causing reduced glucose uptake (33). In this study, F4/80 expressing macrophages were increased in the liver following HFD consumption, and this was reduced by the treatment with AuNPs, demonstrating a direct anti-inflammatory effect. The percentage of F4/80 positive macrophages were not increased by HFD consumption in the abdominal fat tissue. Longer HFD feeding duration may be need to observe increased macrophages in the fat tissue as shown in the other study, while the macrophages are not the only immune cells in the fat causing inflammatory responses (34). We think that the increase in the liver and not in the fat represents different recruitment dynamics in this model. Irrespective of macrophage accumulation, TNFα and upstream TLR-4 mRNA expression were both increased. As such, fat derived adiponectin (insulin sensing promotor) and GLUT4 (insulin dependent glucose transporter) were significantly down-regulated in the HFD-C mice, resulting in glucose intolerance. The up-regulation of ATGL, CPT-1α and leptin in the fat tissue reflects an increase in lipid influx into the adipocytes, while

339 increased ATGL may contribute to nearly doubled blood NEFA levels following HFD consumption.
340 Similar changes in TNFα were seen in the liver, resulting from excessive liver lipid storage which
341 would activate the Kupffer cells (liver macrophage-like cells)(35). This inflammatory response in
342 turn stimulates SREBP-1c which further activates FASN activity to increase lipogenesis (36), leading
343 to a fatty liver (36, 37). This study strongly points to an anti-inflammatory effect by the AuNPs, via
344 suppressing pro-inflammatory cytokine production in both the fat and liver tissues, regardless of the
345 impact on macrophage numbers.
346
347 Interestingly, the changes in metabolic markers were not consistent in the HFD-LAu and HFD-HAu
348 groups, suggesting different working mechanisms. In the HFD-Lau group, increased fat CPT-1α may
349 increase lipid oxidation, resulting in a better blood lipid profile and smaller fat mass (38). Upon AuNP
350 treatment, liver lipogenesis appeared to be suppressed with a synchronized down-regulation of
351 SREBP-1c and FASN mRNA levels. Based on these observations, we propose that low dose AuNP
352 could reduce hepatic ectopic lipid deposition to impede the development of obesity-associated fatty
353 liver disease. In the HFD-HAu group, increases in fat GLUT4 and adiponectin is suggestive of an
354 improved insulin response and glucose uptake. There was a drastic increase in GLUT4 by AuNP
355 treatment in this group, which may contribute to significantly improved glucose clearance during
356 IPGTT.
357
358 The *in vitro* study allowed us to examine the impact of AuNPs on individual cell types, as well as
359 their interactions via the use of a contact co-culture system. Interestingly, AuNPs induced
360 inflammatory responses in macrophages cultured alone as foreign objectives; however this response
361 seemed to be suppressed when grown in the presence of adipocytes. Increased oxidative stress has
362 been suggested to be the major cause of organ toxicity (39). Increased ROS production appeared in
363 macrophages treated with high concentration of AuNP in line with reduced cell viability; however
364 such changes diminished with the co-culture with adipocytes suggesting unknown antioxidative

mechanism due to the interaction between these two cell types. Similarly, AuNP treatment of adipocytes cultured alone did not change their differentiation rate into mature adipocytes, nor metabolic markers. However, it did result in reduced lipid droplet size, which may contribute to slow-down fat accumulation during HFD consumption. On the other hand, AuNP treatment of adipocytes co-cultured with macrophages resulted in metabolic marker change that may potentially improve lipid metabolism as well as glucose uptake. Given that the co-cultured adipocyte and macrophage more closely resembles conditions *in vivo*, this suggests that the same interactions may be occurring within the mice treated with AuNPs. These studies also highlight the limitation of using single-cell culture systems. Additionally, these changes were more prominent at 24h, suggesting daily administration of the AuNPs is desired to exert a continuous and more refined metabolic effect.

Neutralization of circulating TNFα alone has been shown to increase insulin sensitivity and glucose uptake in peripheral tissues, although to date, such approaches have not been successfully translated into humans (27, 30, 31). This is perhaps due to the involvement of other pro-inflammatory cytokines yet to be defined. Therefore, altering macrophage responses may be the key to inhibit systemic inflammatory processes. AuNPs emerge as highly suitable candidates to carry out this task, with both TNFα and TLR-4 down-regulated upon AuNP administration, consistent with our previous acute study in lean mice (6). The uptake and elimination of the gold from tissues is still a key issue when considering long-term treatments. In line with previous studies, AuNPs were taken up into the surrounding abdominal fat after repeated IP administration, which were then able to enter the blood stream, from which they then distribute and accumulate within other organs (6, 40).

Conclusions

In conclusion, the alterations in the local pro-inflammatory cytokine environment by AuNPs may be the key underlying mechanism for the weight reduction in HFD-fed mice. Specifically, AuNP-treated mice were protected against the development of HFD-induced glucose intolerance as well as

hyperlipidemia. AuNPs may serve as a new paradigm to inspire treatments for weight loss and the prevention of obesity-related metabolic disorders and as a useful research tool to probe biological mechanisms.

Declarations

Ethics Approval

All procedures were approved by the Animal Care and Ethics Committee at the University of Technology Sydney (ACEC#2011-403A), and carried out following the Guidelines for the Care and Use of Laboratory Animals of the National Health and Medical Research Council.

Consent for publication N/A

Availability of data and materials

- All data generated or analysed during this study are included in this published article [and its corresponding supplementary information file].

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

HC., JPMN., SMV., conceived and designed the experiments; Performed experiments: H.C., JPMN., KM., YT., DPB, YLC. Analyzed the data: HC., JPMN., KM., YT., DPB, YLC, BO. Contributed reagents/materials/analysis tools: HC., KM., DPB., MBC., BKM., SMV. BO. Wrote the paper: HC., JPMN., SMV., KM., DPB., MBC.
All authors read and approved the final manuscript.

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Figure legends

Figure 1 (a) intraperitoneal glucose tolerance test (IPGTT, glucose 2g/kg), (b) area under the curve (AUC) of the (a), at 8 weeks of treatment. Data are expressed in mean ± S.E.M. IPGTT difference in (a) were analyzed using one-way ANOVA with repeat measures followed by *post hoc* Bonferroni test. * $P< 0.05$, Chow-C and HFD-LAu *vs.* HFD-C at 15 min; † $P< 0.05$, Chow-C and HFD-LAu *vs.* HFD-C at 30 min; ‡ $P< 0.05$, Chow-C, HFD-LAu, and HFD-HAu *vs.* HFD-C at 60 min; γ $P< 0.05$, Chow-C, HFD-LAu, and HFD-HAu *vs.* HFD-C at 90 min. AUC difference in (b) were analyzed using one-way ANOVA followed by *post hoc* Bonferroni test. * *P<0.05 vs.* Chow-C group; † *P<0.05 vs.* HFD-C group; *n*=6.

Figure 2 Retroperitoneal fat and liver mRNA expression of (a, e) TNFα, (b, f) TLR-4, (c, g) and SAA-1 in the Chow-C, HFD-C, HFD-LAu, and HFD-HAu mice at 9 weeks of treatment. The percentage of macrophage number and representative image of macrophage number in the abdominal fat (d) and liver (h) tissues by immunohistochemistry (IHC) staining at the same time point. Results are expressed as mean ± S.E.M, relative to 18s. Data were analyzed by one-way ANOVA followed by *post hoc* Bonferroni test. * *P<0.05 vs.* Chow-C; ** *P<0.01 vs.* Chow-C; † *P<0.05 vs.* HFD-C; †† *P<0.01 vs.* HFD-C. *n*=5-10.

Figure 3 Retroperitoneal fat mRNA expression of (a) GLUT-4, (b) ATGL, (c) CPT-1α, (d) SREBP-1c, (e) adiponectin, (f) FOXO1, and (g) leptin in Chow-C, HFD-C, HFD-LAu, and HFD-HAu mice at 9 weeks of treatment. Results are expressed as mean ± S.E.M, relative to 18s. Data were analyzed by one-way ANOVA followed by *post hoc* Bonferroni test. * *P*<0.05 *vs.* Chow-C; ** *P*<0.01 *vs.* Chow-C; † *P*<0.05 *vs.* HFD-C; †† *P*<0.01 *vs.* HFD-C; *n*=5-10.

Figure 4 Liver mRNA expression of (a) GLUT-4, (b) ATGL, (c) CPT-1α, (d) SREBP-1c, (e) FASN, and (f) FOXO1 in Chow-C, HFD-C, HFD-LAu, and HFD-HAu mice at 9 weeks of treatment. Results

573 are expressed as mean ± S.E.M, relative to 18s. Data were analyzed by one-way ANOVA followed
574 by *post hoc* Bonferroni test. * $P<0.05$ *vs.* Chow-C; ** $P<0.01$ *vs.* Chow-C; † $P<0.05$ *vs.* HFD-C; ††
575 $P<0.01$ *vs.* HFD-C; n=5-10.
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