UNIVERSIDADE PAULISTA – UNIP

PROGRAMA DE PÓS-GRADUAÇÃO MESTRADO/DOUTORADO EM PATOLOGIA AMBIENTAL E EXPERIMENTAL

HYPERLIPIDIC DIET INCREASES FAT WEIGHT AND INDUCES GLUCOSE INTOLERANCE IN BALBC-XID MICE

Dissertação apresentada ao Programa de Pós Graduação em Patologia Ambiental e Experimental da Universidade Paulista – UNIP, para obtenção do título de Mestre em Patologia Ambiental e Experimental.

AUGUSTO CÉSAR BALDASSI

SÃO PAULO
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Aprovado em:

BANCA EXAMINADORA

/ /

Prof. Dr. Mario Mariano
Universidade Paulista - UNIP

/ /

Profª. Drª. Maria Martha Bernardi
Universidade Paulista - UNIP

/ /

Profª. Drª. Miriam Oliveira Ribeiro
Universidade Presbiteriana Mackenzie

DEDICATÓRIA

Dedico esse trabalho ao meu filho Marco Antônio.

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MUITO OBRIGADO.

RESUMO

Objetivo: O camundongo Balbc-XID (XID) é um animal deficiente de célula B_1. Esses animais são mais susceptíveis à obesidade induzida pela dieta além de possuírem maior predisposição a desenvolverem intolerância à glicose. Esse fato ocorre pela ação antiinflamatória que a célula B_1 possui. O objetivo desse estudo foi caracterizar se o animal XID seria um modelo de obesidade e intolerância à glicose ao final da dieta hyperlipídica e normolipídica administradas por 3 meses. **Métodos:** foram avaliados: 1) ganho de tamanho semanal por 3 meses; 2) ganho de peso semanal e o índice de Lee ao final dos 3 meses; 3) peso da gordura retroperitoneal e gonadal após os 3 meses; 4) O tamanho dos adipócitos subcutâneos e 5) Teste de tolerância à glicose ao final das dietas administradas. **Resultados:** Em relação ao animal Balbc-Normal, tratado com ração normolipídica, o animal XID (tratado com ração hiperlipídica) desenvolveu: 1) Ganho de peso e índice de Lee maior; 2) Maior acúmulo de gordura retroperitoneal e gonadal; 3) Maior área e quantidade de adipócitos subcutâneos e 4) Desenvolveu intolerância à glicose. **Conclusão:** o camundongo XID representa um excelente modelo para o estudo dos efeitos da obesidade induzida pela dieta hiperlipídica e intolerância à glicose.

Palavras-chave: obesidade, sobrepeso, modelo animal, camundongo mutante, intolerância à glicose.

ABSTRACT

Objective: The Balbc-XID (XID) mice are a genetically deficient B_1-cell animal. These animals are more susceptible to diet induced obesity and to develop glucose intolerance. These facts are based on the anti-inflammatory role played by B_1-cell. The objective of the present study was to characterize if the XID mice would be a model of obesity and glucose intolerance at the end of the hyperlipidic and normolipidic diet administrated for 3 months. **Methods**: it was evaluated: 1) Weekly lenght gain in the 3 months of administration of hyperlipidic and normolipidic diet; 2) Body weight gain and the Lee Index at the end of the diet; 3) The weight of retroperitoneal and gonadal fat at the end of the diet; 4) The size of subcutaneous adipocytes and 5) Glucose tolerance test at the end of the diet. **Results:** relative to Balbc-WILD mice (WILD) treated with the normolipidic diet, the XID mice treated with hyperlipidic diet shows: 1) increased the body weight gain and the Lee index; 2) the visceral and subcutaneous fat; 3) the number of larger adipocytes and the 4) developed intolerance to glucose. **Conclusions**: the XID mice could represent an animal model to study the effects of hyperlipidic diet-induce-overweight/ obesity and glucose intolerance.

Key words: obesity, overweight, animal model, mutant mice, glucose intolerance.

SUMÁRIO

HYPERLIPIDIC DIET INCREASES FAT WEIGHT AND INDUCES GLUCOSE INTOLERANCE IN BALBC-XID MICE.

Augusto Cesar Baldassi; Leoni Villano Bonamin; Eduardo Fernandes Bondan; Maria Martha Bernardi; Wilton Pereira dos Santos; Flora Cordeiro; Mario Mariano.

Graduate Program of Environmental and Experimental Pathology, Paulista University, Rua Dr. Bacelar, 1212, 04026-002, São Paulo, SP, Brazil.

Running title: Hyperlipidic diet and BALB/c-XID mice.

Correspondence to: Mario Mariano

Graduate Program of Environmental and

Experimental Pathology

Paulista University

Rua Dr. Bacelar, 1212, 04026-002

São Paulo, SP, Brazil

e-mail: mariomu@uol.com.br

e-mail: acbaldassi@hotmail.com

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Highlights

XID mutant mice with lack of B_1-cell, received hyperlipidic diet.

Increased body weight gain, Lee index and fat weigh were observed.

Also glucose intolerance was observed in these mice.

These mice would be a model to study overweight/ obesity.

INTRODUCTION

The incidence of obesity and overweight has increased substantially in recent decades, and obesity is now a major global health problem[1][2][3][4]. The considerable health burden of obesity and overweight has negative effects on many health outcomes, leading to disability, mortality and increased health care use[2]. Individuals who are overweight and obese have an increased risk of several diseases, including Type 2 diabetes[5], respiratory disorders[6], cardiovascular disease[5], endocrine disorders[7], immune dysfunction[8][9], certain types of cancer[10], psychiatric disorders[11] and decreased fertility[12].

Increasing evidence indicates that obesity is causally linked to a chronic low-grade inflammatory state[13] , which contributes to the development of obesity-linked disorders, in particular to metabolic dysfunction[14]. It is now well established that adipose tissue is not only involved in energy storage but also functions as an endocrine organ that secretes many bioactive substances. Although macrophages and T cells have been implicated in this process, emerging evidence suggests that B-cells also modulate obesity-induced adipose tissue inflammation and insulin resistance[15].

The B_1-cell has been considered a cell of the innate and adaptive immune system and that therefore has characteristics of both cellular type, such as phagocytic capacity in addition to production of cytokines and antibodies[16]. Therefore, these cells have an effective participation in inflammatory processes. Because obesity is considered a chronic inflammatory process, a concept based that the circulating levels of many cytokines and acute phase proteins associated with inflammation is elevated in obese patients, it is possible that B_1-cell may be related to obesity[17].

Animal models of obesity may be useful in different areas of science, allowing understanding the underlying mechanisms of obesity and contributing to its treatment. Thus, animal models of obesity and insulin resistance, either those in whom insulin resistance is secondary to a pathological condition or those in whom insulin resistance is induced through the administration of drugs or diets, are very useful for the study of obesity and diabetes and its metabolic consequences[18].

The Balbc-XID mice is a genetically deficient B-cell animal. These animals are more susceptible to experimentally induced diabetes, evidenced by high blood glucose levels in response to streptozocin over C57BL/6W mice. These results suggest a new role for B_1-cell in metabolic processes, particularly in the pathogenesis of diabetes[19].

The objective of this study was to characterize if the Balbc-XID mice would be a model of obesity and insulin resistance at the end of the hyperlipidic and normolipidic diet administrated for 3 months evaluating:1) weekly weight gain in the 3 months of administration of hyperlipidic and normolipidic diet; 2) Lee Index at the end of the diet; 3) The weight of retroperitoneal and gonadal fat at the end of the diet; 4) The area of subcutaneous adipocytes and 5) Glucose tolerance test at the end of the diet.

MATERIAL AND METHODS

Ethics statement

The present study was approved by the Ethics Commission of the University Paulista (protocol no. 050/16 -CEUA/ICS/UNIP). All efforts were made to minimize the suffering of the animals and reduce the number of animals used. The experiments were performed in accordance with good laboratory practice protocols.

Animals

A total of 12 XID male mice and 12 WILD, with 30 days of age at the beginning of experiments were obtained from the facilities of the laboratory of Pesquisas Mutidisciplinares /UNIP. The mice were housed in groups of six in microisolator cages under controlled temperature (24-26°C) and humidity (50-65%) in artificially lit rooms on a 12 h/12 h light/dark cycle (lights on at 7:00 AM) with free access to filtered water and irradiated food (BioBase, Águas Frias, Brazil). Sterilized, residue-free wood shavings were used for animal bedding.

Experimental design

Mice of the hyperlipidic diet (6 XID and 6 WILD mice) were given free access to the diet (purified hyperlipidic diet in pellets- PragSolutions Serviços e Com. LTDA, São Paulo, Brazil; total 45% Kcal). Mice of normolipidic diet (6 XID and 6 WILD mice) received the purified control diet (PragSolutions Serviços e Com. LTDA, pellets, São Paulo, Brazil; total 10% Kcal). The consumption of both diets was performed each three days, during 90 days of treatment. The body weight was evaluated weekly from 30 to 122 days of age and the body weight gain calculated. The nasoanal lengths and the

body weight were used to calculation the Lee indexes between groups. This index was calculated by dividing the cube root of body weight (g) by nasoanal length (cm) and multiplying the result by 1000. At 119 days of age the glucose tolerance test of all mice was performed and at 120 days of age they were weighted, the body length measured and killed. Then, the retroperitoneal and retrogonadal fat were harvest and weighted. These fat measures were divided by the body weight. Portions of the hypodermis were collected to adipocytes histopathology.

Histopathology of adipocytes

A 2x2 cm fragment of abdominal skin, including the hypodermis and abdominal muscle near the umbilical scar, was removed, fixed on a thin piece of paper and immersed in 10% buffered formalin, for fixation. The skin was stained with haematoxylin–eosin and 10 serial photomicrographs were taken from randomly chosen microscopic fields of the hypodermis, using a Nikon (Kanagawa, Japan) E200 microscope (10 x objective) equipped with a Digital Coolpix (Kanagawa, Japan) camera linked to an LCD monitor. The area of each entire adipocyte present in each field was measured, in pixels, using the ImageJ software (National Institutes of Health,Bethesda, MD, USA). In the first analysis of the area of adipose cells, two clearly distinct populations were identified: (1) small cells, with an area measuring < or = 9000 pixels; and (2) larger cells, with an area measuring > 9000 pixels. The areas of both groups of adipocytes were calculated.

Glucose tolerance test (GTT)

To make the Glucose tolerance test (GTT), the steps below were followed:

1 - Deprive animals of water and food for 12 hours.

2 - The first blood collection should be performed after cutting the tail end of the animal, corresponding to time zero (t0) of the test.

3 - Then we give glucose solution to the animals (1g/kg of Dextrox solution made by dissolving 40g in 250ml of water).

4 - After that, the blood collection is made at 30 min (t30), 60 min (t60) and 120 min (t120) from t0.

Statistical analysis

Homoscedasticity was verified using an *F*-test or Bartlett's test. Normality was verified using the Kolmogorov–Smirnov test. Two-way ANOVA followed by Bonferroni's test was used to analyze data with two factors. Unless indicated otherwise, results are expressed as the mean ± SEM. In all cases, results were considered significant at $P < 0.05$.

RESULTS

Fig.1 shows the body weight gain in XID and WILD mice observed during 13 weeks of exposure to normolipidic and hyperlipidic diet. The diet and strains influenced the results with interaction between factors. The post hoc test indicates an increased weight gain in all sessions in WH (WILD hyperlipidic) and XIDH (XID hyperlipidic) relative to WN (WILD normolipidic) and XIDN (XID normolipidic).

Fig.2 shows the bodyweight length (2A) and Lee index (2B) in XID and WILD mice. The diet and strain of mice did not influence the results of body length gain. Relative to body weight gain, the diet influenced the results but no differences were observed between strains with no interaction between factors. The body weight gain was significantly increased in XIDH group relative to XIDN mice.

Concerning the Lee index, the diet and strain influenced the results with interaction between factors. The post hoc test indicates a decreased Lee index in XIDN mice relative to WN group. The statistical data are show in the supplementary table 1.

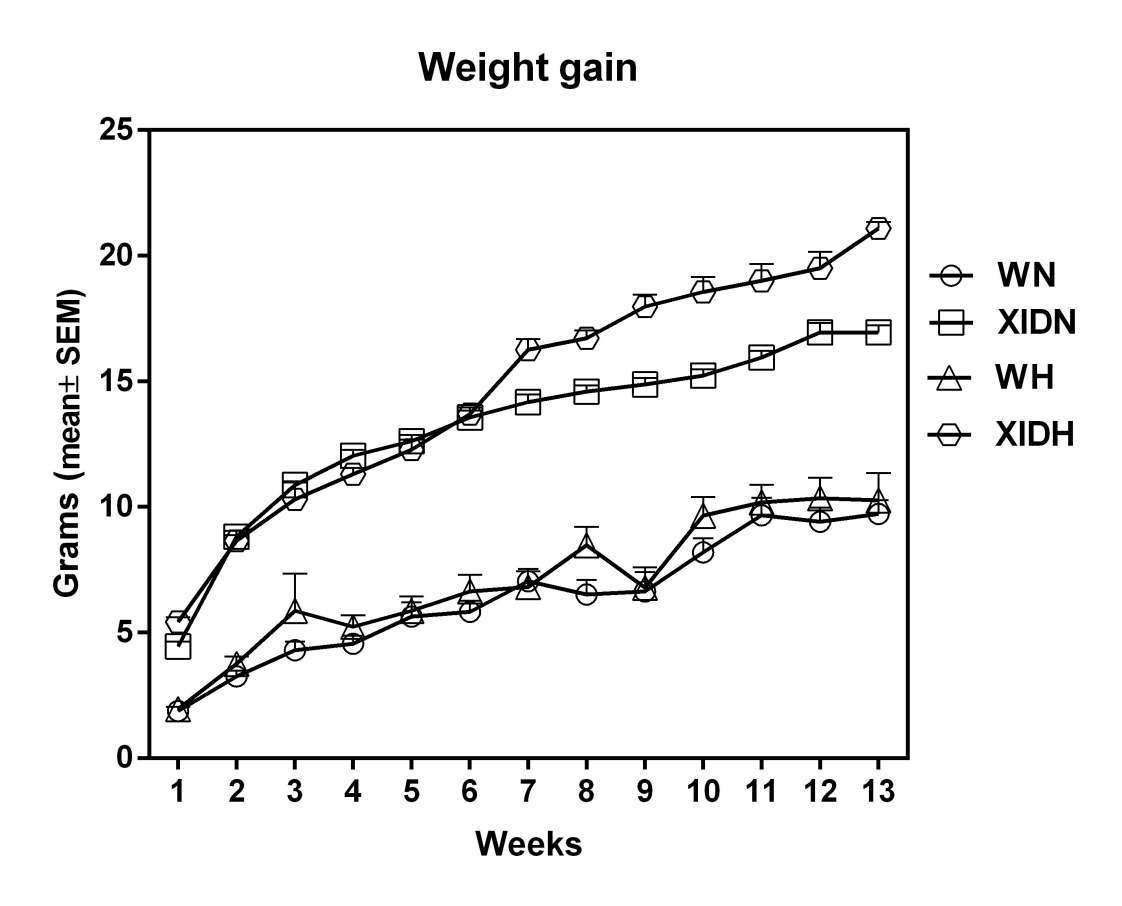

Fig.1. Bodyweight gain of WILD and XID mice treated with normolipidic and hyperlipidic diet. N= 6 / group. Two way ANOVA followed by the Bonferroni test. **p< 0.01 relative to the respective control group or between strains. The data are expressed as the mean ± SEM.

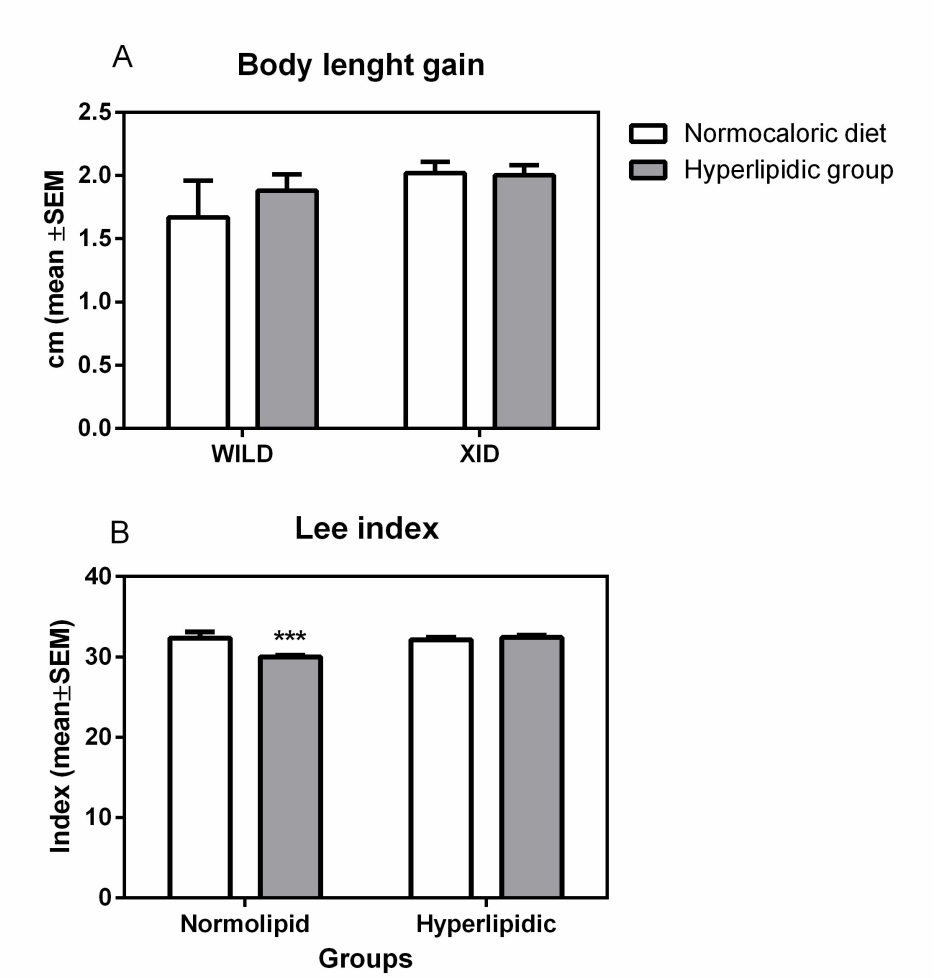

Fig.2. Bodyweight length (A) and Lee index (B) of WILD and XID mice treated with normolipidic and hyperlipidic diet. N= 6 / group. Two way ANOVA followed by the Bonferroni test. **p< 0.01 relative to the respective control group or between strains. The data are expressed as the mean ± SEM.

Fig.3 show the retroperitoneal (3A) and gonadal (3B) fat / body weight of the XID and WILD mice. In retroperitoneal fat, the diet influenced the results but not the strain without interaction between factors. The post hoc test indicates an increased in retroperitoneal fat in XIDH relative to XIDN. The gonadal fat was influence by the diet with interaction between factors. The post hoc test indicates increased the gonadal fat in WH and XIDH relative respectively to WN and XIDH mice. The statistical data are show in the supplementary table 1.

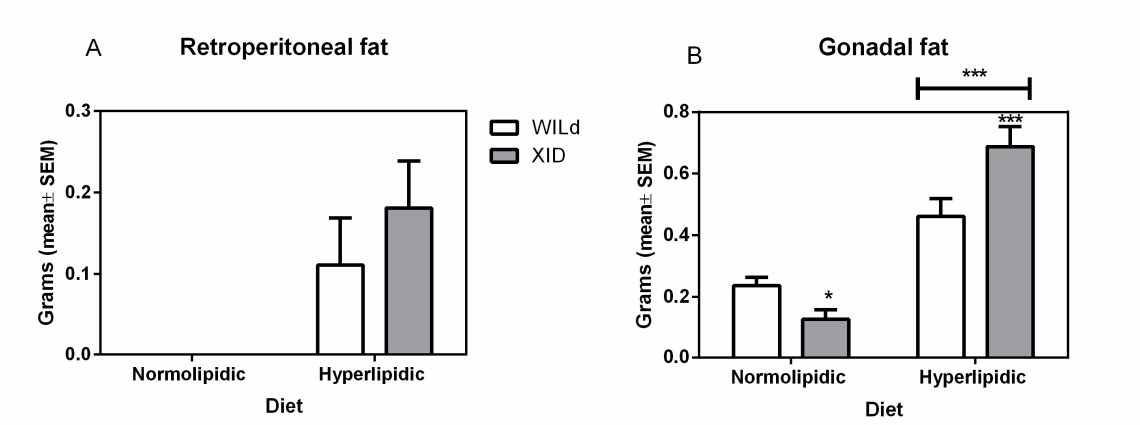

Fig.3. Retroperitoneal (A) and Gonadal (B) fat/body weight of WILD and XID mice treated with normolipidic and hyperlipidic diet. N= 6 / group. Two way ANOVA followed by the Bonferroni test. **p< 0.01, ***p 0.001 relative to respective control group. The data are expressed as the mean ± SEM.

Fig.4 shows the total adipocytes area (4A), the larger (4B) and small (4C) adipocytes area of the XID and WILD mice. Relative to the total adipocytes area the two-way ANOVA indicates significant differences of the diet and strains without interaction between factors. Both mice strains treated with hyperlipidic diet showed increase in total adipocytes area relative to the respective control groups. These differences were also seen in both classes of adipocytes (small and large ones), differently from the data previously described by our group, when specific transgenerational patterns were observed after offering caloric diet[9][20]. The statistical data are show in the supplementary table 1.

The glucose levels were depicted in fig.5. The two way ANOVA show differences relative to diet and strains without interaction between factors. Relative to WN group, the WH group presented high levels of glucose at 30 min, the XIDN group at 30 and 60 min and the XIDH group at 30, 60 and 120 minutos. Comparison between WH and XIDH groups revealed that the XIDH mice had high glucose levels at 0, 60 and

120 min than WH group. No differences were detected in glucose levels of XIDN and XIDH groups. Statistical data are show in the supplementary table 2.

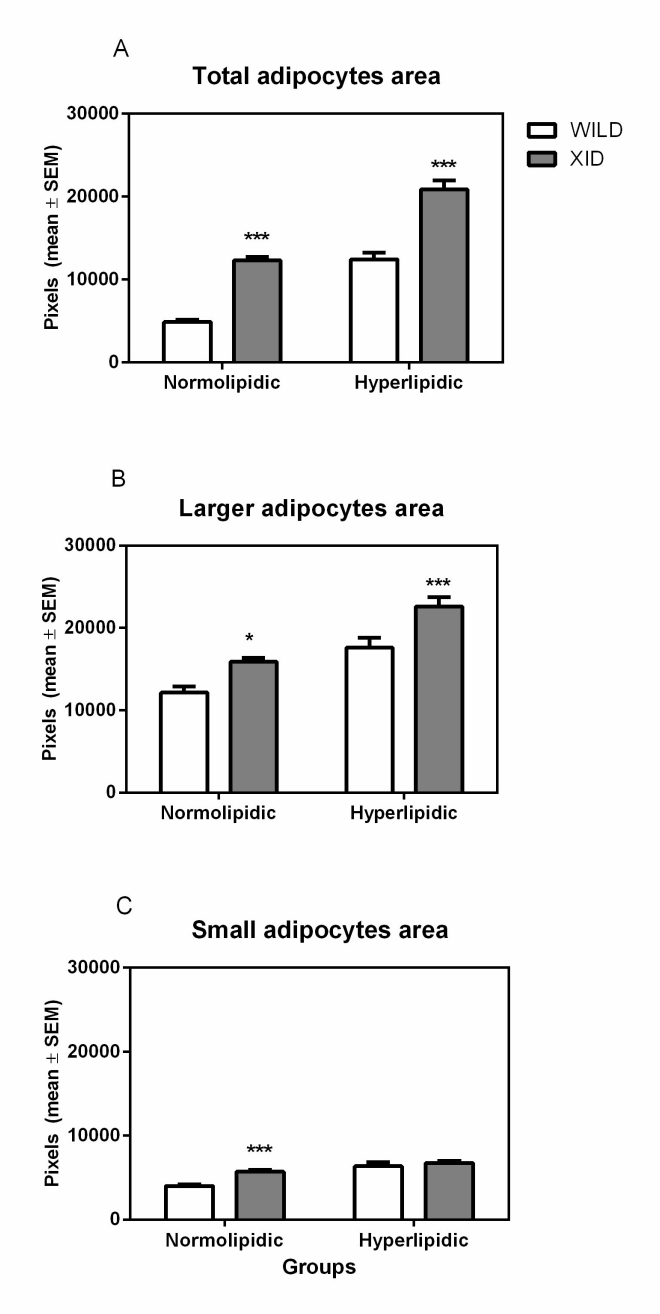

Fig.4 Total adipocytes area (A), larger adipocytes area(B) and small adipocytes area (C) of WILD and XID mice treated with normolipidic and hyperlipidic diet. N= 20/ group. Two way ANOVA followed by the Bonferroni test. ***p 0.001 relative to the respective control group or between strains. The data are expressed as the mean ± SEM.

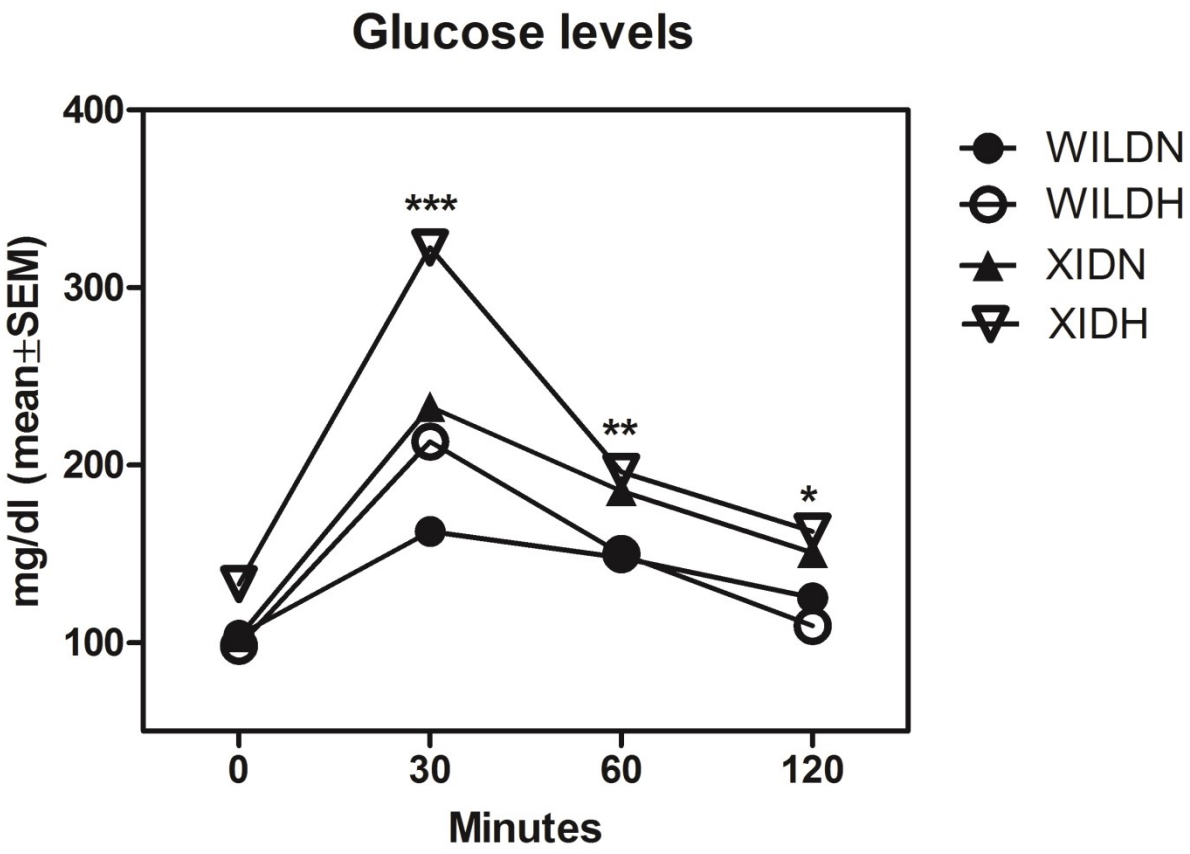

Fig.5. Glucose levels of WILD and XID mice treated with normolipidic and hyperlipidic diet. N= 6 / group. Two way ANOVA followed by the Bonferroni test. **p< 0.01, ***p 0.001 relative to WILD group. Relative to WN group, the WH group presented high levels of glucose at 30 min (p< 0.01), the XIDN group at 30 (p< 0.001) and 60 (p< 0.01) min and the XIDH group at 30 (p< 0.001), 60 (p< 0.001) and 120 (p< 0.05) minutes. The data are expressed as the mean ± SEM.

DISCUSSION

Lee et al[21] derived a formula for rodents from a nutritive index for children (Von Pirquet, 1917, apud[22] the Lee index -weight $^{0.33}$/Naso-Anal Length- as a simply subjective assessment of the nutritive state of the animal). Despite Lee did not measured the fatness of his rats, the Lee index is still used as an index of obesity in rodents[23]. Moderate obesity in rats has been defined as values of 10-25% greater body weight than controls whereas values greater than 40% indicate severe obesity. Thus, according the Lee index, the XIDH mice showed similar results relative to XIDN mice. However, taken the increase in Lee index between XIDN and XIDH was about 6-7% whereas between WN and WH was 1-2 %. These data suggest a tendency to obesity/overweight in XID mice.

Data of body weight gain revealed that the hyperlipidic diet increased bodyweight gain during the weeks of treatment in XID mice. Interesting XID mice fed with normolipidic also presented increased body weight during the experiments relative to WN and WH groups suggesting a natural tendency to obesity/overweight.

However, obesity as defined by an excessive accumulation of fat[24] is not always associated with a high bodyweight[25] or the Lee index[25]. Body weight gain and the Lee index did not accurately identify obese from non-obese individuals[26]. It is postulated that the reason for the lack of correlation between obesity and the Lee index in intact rats is that length is a relatively weak predictor of fat free mass in animals of a similar age and nutritional history[25].

Therefore, an objective measure of adiposity should always be used as the criterion for obesity. In the present study, the XIDH mice showed increased levels of retroperitoneal and gonadal fat, many times higher than their WILD control group

treated with hyperlipidic diet, indicating a high accumulation of visceral fat. Also, an increase in total adipocytes area was showed in the XIDH mice relative to its control group. Besides this, the increase of adipocyte area was equally increased despite of the adipocyte class[9][27], showing that in XIDH mice there is no trend to change the proportion between cell subtypes, but only the metabolic capacity of retain fat reserves.

The increased weight gain, on Lee index and in the adiposity in XID mice treated with hyperlipidic diet suggests that the lack of B_1-cell predispose XID mice to overweight/obesity.

Based on the analysis of fat distribution obesity is classified as visceral fat obesity and subcutaneous-fat obesity[28]. Metabolic and circulatory disturbances are far more frequently associated with visceral fat obesity than with subcutaneous fat obesity[29]. The fat accumulation in the intra-abdominal cavity is more frequently accompanied by disorders of glucose and lipid metabolism[30][31] and also with hypertension[32], than subcutaneous fat obesity. The intra-abdominal fat has high activities of both lipogenesis and lipolysis and its accumulation induces a high content of free fatty acids which , by portal circulation that goes into the liver directly[33]. Excess free fatty acid may cause the enhancement of lipid synthesis and gluconeogenesis as well as insulin resistance, resulting in hyperlipidemia, glucose intolerance and hypertension and finally atherosclerosis[34].

The results observed in the glucose levels are in according to development of glucose tolerance. Indeed, the XIDH mice show increased levels of glucose in all times of measures after administration.

Thus, the increased of visceral fat weight of XIDH mice could be postulate, at least in part, responsible by the glucose tolerance here observed.

Adipose tissue is a key endocrine organ as it releases multiple bioactive substances, known as adipose-derived secreted factors or adipokines, that have proinflammatory or anti-inflammatory activities[35]. The deregulated expression of these factors, caused by excess adiposity and adipocyte dysfunction, has been linked to the pathogenesis of various disease processes through altered immune responses[36]. As such, much attention has been paid to developing a better understanding of the immunoregulatory functions of adipose tissue. New factors secreted by adipose tissue have been identified that either promote inflammatory responses and metabolic dysfunction or contribute to the resolution of inflammation and have beneficial effects on obesity-linked metabolic disorders[37]. Various inflammatory responses are dynamically regulated in adipose tissues and most of the immune cells in adipose tissues are involved in obesity-mediated metabolic complications, including insulin resistance[38].

B_1-cells are a sub-class of B cell lymphocytes that are involved in the humoral immune response[39]. They are considered part of the adaptive and innate immune system, based on the fact that B_1-cell perform many of the same roles as other B- cells: making antibodies against antigens and acting as antigen presenting cells[40] and also have the ability to became phagocyte cells. They were first characterized in 1983 and express the phenotype $IgD^{low}IgM^{hi}CD23\text{-}CD19+CD11b+$ whilst conventional B cells are identified by the phenotype $IgD^{hi}IgM^{low}CD23^{+}CD19^{+}CD11b^{-}CD5^{-}$. Further, B_{1a}-cell differ from B_{1b}-cell by the expression of CD5 molecules in the former[42]. In addition to the promiscuous expression of markers for lymphoid and myeloid cell lineages, added with the CD5 T cell marker, these cells have also a peculiar distribution in the body's economy of mice: they are predominantly found in pleural and peritoneal cavities, being few in the spleen and almost absent in lymph nodes[43]. Contrary to the

bone marrow origin of conventional B-cells, B_1-cell is long-lived and auto-renewing cells[44].

Thus, the increased in visceral and subcutaneous fat in XID mice after hyperlipidic diet also suggest that the lack of B_1-cell can be involved in the chronic inflammatory processes observed in overweight/obesity. The B_1-cell play an anti-inflammatory role in obesity through a combination of IG-M and IL-10 production, besides that, B_1-cell also attenuate the participation of M1 macrophages, as M1 macrophages are rare in lean adipose tissue. B_1-cell derived IG-M can reduce inflammation and promote apoptotic cell clearance, process that become dysregulated during the progression of obesity[15].

The participation of B_1-cell in a murine model of spontaneous diabetes has been reported[45][46]. Alvares-Saraiva et al (2015) describe the role of B_1-cell in streptozotocin induced diabetes in mice. These authors demonstrated that XID mice are more susceptible to streptozotocin treatment than WILD mice, as evidenced by their higher blood glucose level in response to streptozotocin. These data explain the tolerance to glucose here observed in XIDH mice and suggest that B_1-cell is involved with insulin control.

CONCLUSIONS

Finally, the present data strongly suggest that the XID mice could represent an animal model to study the effects of hyperlipidic diet-induce- overweight/ obesity and insulin resistance. Besides that the data also provides the evidence that B_1-cell attenuate diet-induced glucose intolerance in mice.

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Supplementary table 1. Statistical data . Two way ANOVA.

Parameter	Interaction	Diet	Strains
Body length gain	$F_{(1,20)} = 0.44$ P=0.51	$F_{(1,20)}=0.33$ P=0.57	$F_{(1,20)} = 1.95$ P=0.18
Weight gain	$F_{(1,20)} = 4.24$ P= 0.06	$F_{(1,20)} =9.72$ P = 0.005	$F_{(1,20)} = 0,04$ P= 0.84
Lee index	$F_{(1,20)} =8.35$ P =0.009	$F_{(1,20)} = 5.98$ P =0.02	$F_{(1,20)} = 5.45$ P = 0.03
Retroperitoneal fat	$F_{(1,20)}=1.34$ P =0.26	$F_{(1,20)}=12.09$ P =0.002	$F_{(1,20)} = 1.34$ P = 0.20
Gonadal fat	$F_{(1,20)}=8.60$ P =0.008	$F_{(1,20)}=58.40$ P < 0.0001	$F_{(1,20)} = 3.35$ P = 0.08
Total adipocyte area	$F_{(1,76)} = 0.49$ P = 0.49	$F_{(1,76)} = 127.76$ P < 0.0001	$F_{(1,76)} = 123.35$ P < 0.0001
Larger adipocyte area	$F_{(1,76)} = 0.44$ P = 0.51	$F_{(1,76)} = 41.25$ P < 0.0001	$F_{(1,76)} =21.30$ P< 0.0001
Small adipocyte fat	$F_{(1,76)} = 4.65$ P = 0.03	$F_{(1,76)} = 33.14$ P < 0.0001	$F_{(1,76)} = 12.68$ P < 0.0006

Table 2. Statistical data of glucose levels. Bonferroni test.

Groups/min	WN	WH	XN	XH
30 minutes				
WN		***	***	ns
WH	***		ns	ns
XN	***	ns		ns
XH	***	-	ns	
60 minutes				
WN		ns	*	***
WH	*		*	**
XN	*	*		ns
XH	**	**	ns	
120 minutes				
WN		ns	ns	*
WH	ns		**	***
XN	*	***		ns
XH	*	***	ns	

ns= no significant; * p< 0.05; **p< 0.01; ***p< 0.001

CONSIDERAÇÕES FINAIS

1) Modelos animais XID são excelentes para o estudo de sobrepeso/obesidade.
2) Modelos animais XID são excelentes para o estudo de intolerância à glicose.
3) A obesidade é um processo inflamatório crônico, que gera inúmeras alterações na homeostasia orgânica.
4) A célula B_1 possui uma atuação antiinflamatória no mecanismo da obesidade.