Entomology

The impact of chloroquine-induced autophagy inhibition on the metabolic processes of the fat body in *Bombyx mori* during larval-pupal metamorphosis

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Abstract

The fat body of insects is integral to various metabolic processes, including nutrient transformation, energy storage, and the synthesis of hemolymph proteins. This organ also stores carbohydrates primarily in the form of glycogen and mobilizes them as trehalose, while triglycerides serve as a significant energy reserve. Trophocytes, the principal cells of the fat body, are responsible for its metabolic functions and undergo considerable remodeling during the larval-pupal metamorphosis. Autophagy, particularly macroautophagy, is essential for cellular homeostasis, and any disruption in this process can result in metabolic disorders. In insects, especially during metamorphosis, autophagy is critical for the remodeling of the fat body; however, its effects on fat body metabolism have not been thoroughly investigated. This study aimed to explore the role of autophagy in the metabolic functions of the silkworm, *Bombyx mori* fat body during larval-pupal metamorphosis, using chloroquine as an autophagy inhibitor. Our findings elucidate the impact of chloroquine treatment on key components of the fat body and provide insights into the role of autophagy in regulating fat body metabolism.

Keywords: autophagy, *Bombyx mori*, chloroquine, fat body

Introduction

Metabolism encompasses a series of biochemical reactions that are essential for sustaining life in living organisms and serves three fundamental functions: the conversion of food into energy to maintain cellular processes; the transformation of food into building blocks for the synthesis of proteins, lipids, nucleic acids, and carbohydrates; and the elimination of nitrogenous waste (Saghir & Ansari, 2014) [37]. In insects, the fat body is the primary organ that performs all these functions. Furthermore, the fat body plays a role in ecdysis by supplying the requisite energy and hydrocarbons (Roma et al., 2010) [35]. This multifunctional organ is also responsible for the synthesis of hemolymph proteins, which are secreted into the hemolymph in a specific temporal sequence during developmental stages and metamorphosis. In *Bombyx mori*, significant hemolymph proteins include lipophorins (230 kDa), female-specific vitellogenins (178 kDa and 42 kDa), storage proteins (72 kDa–76 kDa), 30K proteins, and several unidentified proteins (Hyrsl & Simek, 2005) [18]. Lipophorin, a multifunctional lipid transport protein, enables the transport of a variety of lipids, including diacylglycerol (DAG), phospholipids, hydrocarbons, cholesterol, and carotenoids throughout the insect's life cycle. Insect hexamers, also called storage proteins, exist as approximately 450 kDa oligomers and 75–80 kDa subunits (Telfer & Kunkel, 1991) [40]. These proteins are present in the hemolymph during the fifth larval stage and are absorbed by the fat body just prior to the pupal stage where they are utilized to construct new structures such as somatic tissues and female reproductive organs (Nagata & Kobayashi, 1990) [31]. 30K proteins are a group of species-specific proteins in Lepidoptera (Kawooya and Law, 1983) [21]. Previous research has demonstrated that 30K proteins serve various functions, including providing essential nutrients for embryogenesis (Zhong et al., 2005) [53], acting as a defense mechanism against fungal infections (Ujita et al., 2005) [44], and effectively inhibiting apoptosis in both human and insect cells (Kim et al., 2003) [22].

Carbohydrates within the fat body are predominantly stored as glycogen and are primarily mobilized in the form of trehalose (Thompson, 2003) [41]. The correlation between glycogen levels and hemolymph trehalose concentration in insects and the role of glycogen phosphorylase in trehalose synthesis reflect the dynamic nature of carbohydrate metabolism (Yamada et al., 2018) [51]. Lipids, another crucial component of fat body, serve a significant role as energy reserves for various physiological processes, including growth, development, flight, migration, diapause, embryonic nutrition, sex pheromone synthesis, cuticle formation, and defensive secretions. The mobilization of lipids, which is stimulated by starvation and elevated physical activity, entails the conversion of triglycerides into energy through the beta-oxidation of fatty acids (Athenstaedt & Daum, 2006) [2]. Lipid storage varies with developmental stages and nutritional status, increasing during feeding periods and decreasing during starvation, oogenesis, or prolonged flight activities (Butterworth et al., 1965) [5].

Trophocytes, the principal cells of the fat body, play a crucial role in the metabolic functions of this tissue, with their composition varying according to developmental stage and nutritional status (Chapman, 1998) [6]. These cells are organized into thin layers or ribbons that facilitate interactions with various tissues and organ systems to support efficient metabolic processes (Dean et al., 1985) [10]. In the final larval stage of *Bombyx mori*, the fat body is characterized by a compact and well-organized structure; however, it undergoes a transformation into a loosely organized nodular configuration during the larval-pupal metamorphosis. This process, referred to as 'fat body remodeling,' entails the mobilization of lipids and proteins and is regulated hormonally (Dean et al., 1985, Larsen, 1976, Li et al., 2019) [10, 27, 30].

Autophagy is a vital cellular recycling mechanism that includes macroautophagy, microautophagy, and chaperone-mediated autophagy, with macroautophagy being the most predominant form. In the process of macroautophagy, double-membrane autophagosomes fuse with lysosomes to create autolysosomes, where cellular components such as damaged proteins and organelles are degraded (Levine & Klionsky, 2004) [29]. The regulation of this process is mediated by autophagy-related (Atg) genes, of which over 30 have been identified in yeast and many of which are evolutionarily conserved (Weidberg et al., 2011) [49]. Disruption of autophagy has been implicated in various metabolic disorders in mammals, including sarcopenic obesity, insulin resistance, and type 2 diabetes mellitus (Kitada & Koya, 2021) [24].

Numerous pharmacological inhibitors of autophagy have been extensively utilized to elucidate the protective and detrimental effects associated with this cellular process. Among these inhibitors, chloroquine, a lysosomotropic weak base, functions by neutralizing intralysosomal acidity, thereby halting autophagy by preventing the fusion of autophagosomes with lysosomes. Initially employed as an antimalarial agent, chloroquine is now recognized as an inhibitor of autophagic flux due to its capacity to elevate lysosomal pH and inhibit the activity of resident hydrolases. Consequently, it has been widely applied as an autophagy inhibitor in both cell culture and in vivo studies (Cynober et al., 1988) [8]. In insects, particularly during the larval-pupal metamorphosis, autophagy-mediated changes encompass the elimination of larval structures and the remodeling of the fat body. Although the role of autophagy in fat body remodeling is well-documented (Zheng et al., 2016) [52], its specific effects on fat body metabolism remain inadequately investigated. This study aims to elucidate the role of autophagy in the metabolic functions of the *Bombyx mori* fat body during larval-pupal metamorphosis, utilizing chloroquine as an autophagy inhibitor. Our findings underscore the impact of autophagy disruption on critical components of fat body in *Bombyx mori*.

Materials and methods

Animals

Japanese × Chinese hybrid strains of the silkworm, *Bombyx mori* were reared on fresh mulberry leaves under controlled environmental conditions of 25±1°C, 75–80% relative humidity, and a 12:12 light-dark photoperiod. The silkworms displayed wandering behavior on day 7 of the fifth instar, and larvae in this final larval stage were used for the experiments.

Chloroquine Injection.

Chloroquine was administered at two distinct dosages (1 mg and 3 mg) on day 7 of the fifth larval instar. The groups receiving 1 mg and 3 mg of chloroquine are designated as Group I and Group II, respectively. Fat body samples from both control and treatment groups were collected every 48 hours, commencing on day 8 of the last larval instar. The control group exhibited cocoon spinning for a duration of three days, which corresponds to the prepupal stage. On day 10 of the final larval instar, the insects transitioned to pharate pupae, with larval-pupal ecdysis occurring on day 11. In the treatment groups, while cocoon spinning was observed, pupation did not take place. Consequently, the developmental stages sampled in the treatment groups were shown as days 10 and 12 of the fifth instar.

Morphological analysis and histochemistry

Fat bodies from both control and treated insects were subjected to histological and histochemical analyses. The tissues were fixed using Bouin's fixative and subsequently dehydrated through a series of ascending alcohol concentrations. Following embedding in paraffin blocks, 5 µm thick sections were stained with hematoxylin and eosin to evaluate the general histological structure. Alcian blue-periodic acid Schiff (PAS) and mercuric bromophenol blue stains were employed to assess carbohydrate and protein content, respectively. The lipid content was determined through oil red staining on 5 µm thick cryostat sections. The tissue sections were examined and photographed using a Zeiss Axio-Imager microscope.

Determination of lipid, glycogen, sugar, and total protein concentrations

Lipids, glycogen, and total sugars were quantified utilizing established biochemical protocols (Van Handel, 1985a; Van Handel, 1985b; Van Handel and Day, 1988; Kaufmann and Brown, 2008) [46, 47, 48, 20]. Fat bodies were homogenized in 50 µl of 2% sodium sulfate and 450 µl of a chloroform-methanol mixture (1:2). The homogenate was subsequently centrifuged at 3000 rpm for one minute, and the resulting pellet was employed for glycogen analysis. To isolate sugars, 0.6 ml of distilled water was added to the supernatant, which was then vortexed and centrifuged at 3000 rpm for one minute. The upper phase was utilized for sugar analysis, while the lower phase was designated for lipid analysis. Samples were subjected to a water bath at 96°C for 15 minutes to facilitate solvent evaporation. For the assays of sugar and glycogen, anthrone reagent was applied, and the tubes were incubated at 96°C for an additional 15 minutes. Absorbance was measured at 625 nm using a spectrophotometer and compared against glucose standards. For lipid analysis, sulfuric acid was introduced to the tubes, which were then incubated at 96°C for 15 minutes, followed by the addition of vanillin-phosphoric acid reagent. Absorbance was measured at 525 nm using an Agilent Cary 60 UV-Vis spectrophotometer and compared with standards prepared from corn oil.

The total protein content was quantified using the Bradford method (Bradford, 1976) [4]. Fat body samples were homogenized in a 0.9% NaCl solution, and the resulting homogenate was subjected to centrifugation at 16,000 g for 10 minutes at a temperature of +4°C. The supernatant obtained was employed for protein quantification, with bovine serum albumin (BSA) utilized as a standard. Absorbance values for both samples and standards, which were incubated with Bradford reagent for 5 minutes at room temperature, were measured at a wavelength of 595 nm using an Agilent Cary 60 UV-Vis spectrophotometer.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted utilizing a discontinuous buffer system as described by Laemmli (1970) [26], employing a Bio-Rad Protean II xi Cell electrophoresis apparatus to evaluate the relative abundance of major protein fractions in fat body samples. All solutions, buffers, and gels were prepared in accordance with the manufacturer's instructions. The separation was performed using 10% gels, while stacking was achieved with 4% gels. Protein samples (7.5 µg) were loaded into the wells and subjected to electrophoresis at 50 V overnight. Thermo Fisher markers (26619) were utilized to ascertain the molecular weights of the protein bands. The gels were subsequently stained with silver nitrate and analyzed using the Chemidoc imaging system (Bio-Rad, Hercules, California, USA).

Lysotracker staining

Fat bodies from both control and treated groups were washed with insect physiological saline and subsequently permeabilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100. The tissues were stained with Lysotracker Red DND-99 (Thermo Fisher Scientific Inc.), diluted at a ratio of 1:1000 in PBS, for a duration of 5 minutes. Following this, the samples were rinsed twice with PBS over a period of 20 minutes and then coverslipped using Mowiol 4-88. Counterstaining was conducted with DAPI. The tissues were examined and photographed utilizing a Zeiss Axio-Imager microscope.

Acid phosphatase assay

The activity of acid phosphatase in fat bodies was assessed utilizing p-nitrophenol phosphate as a substrate, in accordance with the methodology established by Bergmeyer and Bernt (1974) [3]. The optical density of the liberated p-nitrophenol was quantified at a wavelength of 405 nm using a spectrophotometer.

Statistical analysis

PASW Statistics 18 was utilized for the analysis of the data. Given that the sample sizes in the experiment were fewer than 50, the Shapiro-Wilk test was employed to evaluate the normality of the data. For groups that exhibited a normal distribution, an independent t-test was conducted, whereas the Mann-Whitney U test was applied to groups that did not conform to a normal distribution. Statistical significance was established at a threshold of $P < 0.05$. Differences between groups with $P < 0.05$ were deemed statistically significant and are indicated with an asterisk (*) in the graphs.

Results

In the control group larvae, the prepupal stage began on day 7 of the fifth larval stage following gut purge and persisted for a duration of three days. Larval-pupal ecdysis occurred on day 11. However, following chloroquine injections, while cocoon spinning was noted, larval-pupal ecdysis was significantly inhibited (Figure 1A). The overall morphology of the insects across all experimental groups was shown in Figure 1A.

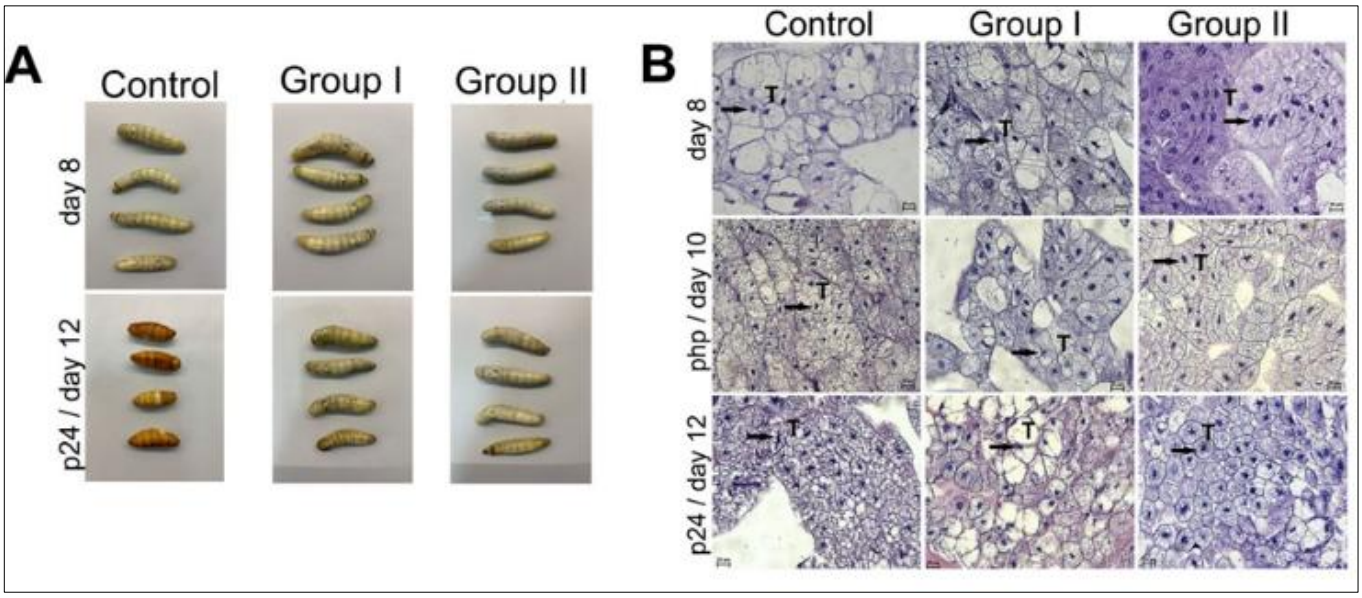

Fig 1: General morphology of control and treatment group insects (A). 1 mg chloroquine treatment group (Group I); 3 mg chloroquine treatment group (Group II). Since larval-pupal molting did not occur in the treatment groups, the sampled developmental stages are represented as the and days 10 and 12 of the fifth instar. The histological structure of the fat body in both the control and treatment groups of insects was assessed using hematoxylin and eosin staining (B). Day 8 of the 5th larval stage (day 8), 24th hour of the pupal stage (p24) in the control group, day 12 of the 5th larval stage (day 12) in the treatment groups. Trophocytes (T), nuclei (arrow).

Histological analysis using hematoxylin-eosin staining demonstrated significant differences in the structural organization of the fat body (Figure 1B). In the control group, trophocytes exhibited well-organized layers characterized by distinct intercellular boundaries and observable nuclear structures. The eosinophilic nature of these cells progressively increased until the pupal stage, indicating an enhancement in cytoplasmic content density. By 24 hours into the pupal stage, the intercellular boundaries in the control group became less distinct due to the increased density of cellular content and a relatively loose, nodular organization. The larval fat body undergoes remodeling rather than complete elimination in *Lepidoptera*, thus this morphological transformation was attributed to the remodeling processes that occur during larval-pupal metamorphosis. Conversely, in the chloroquine-treated groups, the fat body retained characteristics typical of the larval stage, suggesting a delay or inhibition in the normal developmental transition (Figure 1B).

The primary organic constituents of the fat body were assessed utilizing immunohistochemical techniques and spectrophotometric analyses in both the control and treatment groups. The carbohydrate content in the fat bodies of both control and treatment groups was evaluated through Alcian blue-periodic acid Schiff (PAS) staining (Figure 2a) and spectrophotometric analysis of both glycogen and total sugars (Figure 2a, c). In the control group, carbohydrates were found to be abundant in the cytoplasm of trophocytes and the extracellular matrix up to the pupal stage. During the early pupal stage, the intensity of staining exhibited variability; certain trophocytes displayed intense staining, while others exhibited weak staining (Figure 2a). This observed heterogeneity implied that different types of trophocytes may possess specialized functions in glycogen storage. Spectrophotometric analysis indicated a decline in glycogen content with the onset of the pupal stage in the control group (Figure 2b). Following treatment with chloroquine, the intensity of Alcian-PAS staining in both treatment groups was similar to that of the control group until day 12. However, a marked decrease in staining intensity was observed on the final day of the experiment (Figure 2a). The spectrophotometric data obtained supported the histochemical staining results (Figure 2b).

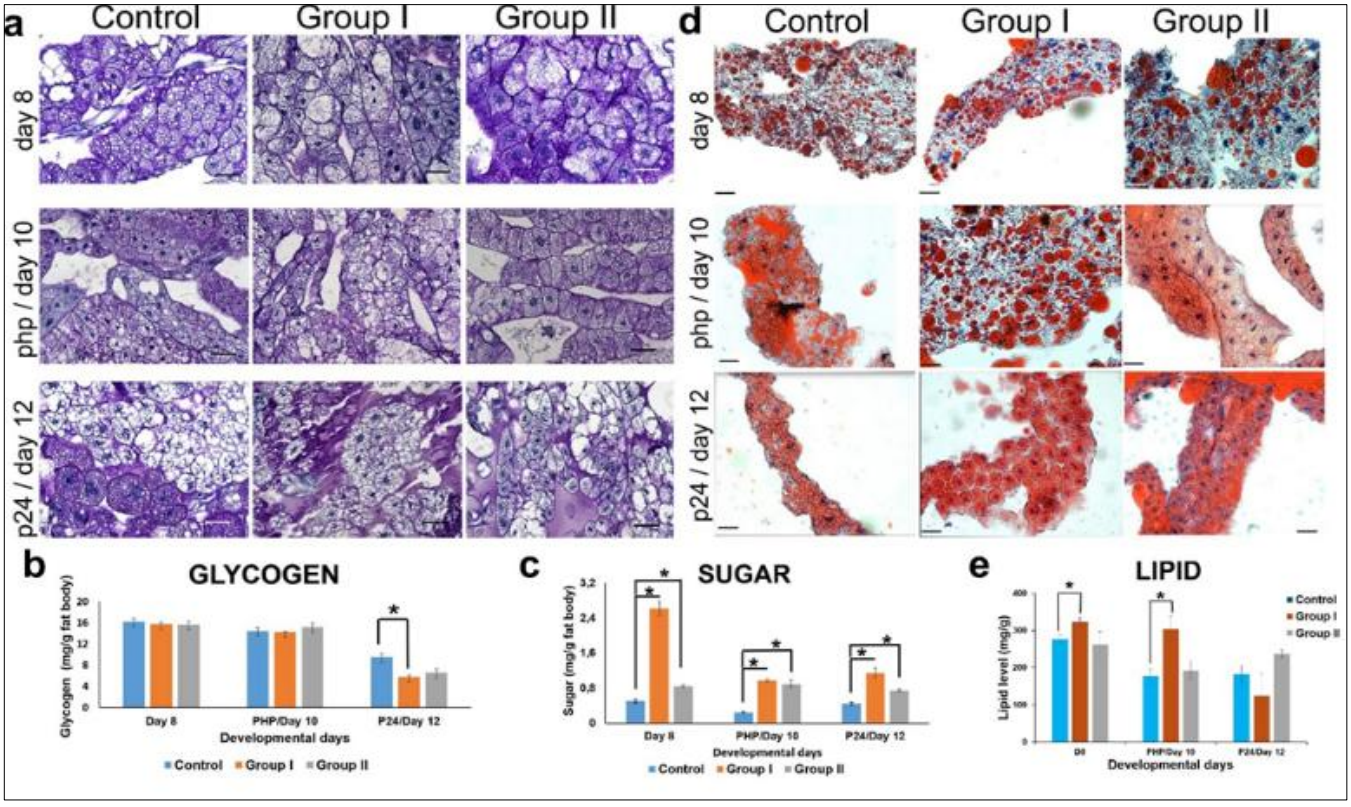

Fig 2: The carbohydrate content in the fat bodies of both the control and treatment groups was evaluated using Alcian blue-periodic acid Schiff (PAS) staining (a) and spectrophotometric analysis of glycogen (b) and sugar (c). The lipid content in the fat bodies of both the control and treatment groups was histochemically assessed using Oil Red O staining (d), while lipid quantities were quantified through spectrophotometric analysis (e). Day 8 of the 5th larval instar (day8), pharate pupal stage in the control group (PHP/ day 10 of the 5th instar in the treatment groups (day10), 24 hours of the pupal stage (P24) in the control group/day 12 of the 5th larval stage (day 12) in the treatment groups. Mean ± standard deviation is indicated on the bars, and significant differences are marked with an asterisk ($p \leq 0.05$).

In addition to assessing glycogen levels, total sugar concentrations in fat bodies were measured using spectrophotometric methods. Notably, the total sugar levels in the fat bodies of both treatment groups were significantly elevated compared to those detected in the control group throughout the duration of the experiment. (Figure 2c). Lipids constitute another significant category of organic components found within the fat body. The lipid content of the fat bodies of both the control and treatment groups was evaluated histochemically using Oil Red O staining (2d) and the amounts of lipid were subsequently measured by spectrophotometric analysis (2e). In the control group, the highest intensity of Oil

Red O staining was observed on day 8 of the fifth larval stage, followed by a decrease from the pharate pupal stage onwards (Figure 2d). The spectrophotometric analysis revealed that the lipid level, which was elevated on day 8 in the control group, declined in the pharate pupa and remained at this level at the onset of the pupal stage (Figure 2e). Fat bodies obtained from Group I, exhibited intense Oil Red O staining on days 8 and 10 of the 5th larval stage. On day 12, a significant decrease in staining intensity was observed. In fat bodies obtained from Group II, the intense staining observed on day 8 decreased significantly on days 10 and 12 (Figure 2d). The findings obtained from histochemical detection of lipids were also supported by spectrophotometric measurements (Figure 2e).

Protein accumulation in the fat bodies was evaluated histochemically through mercury bromphenol blue staining (Figure 3A) and spectrophotometrically utilizing the Bradford method (Figure 3B). In the control group, a significant increase in staining intensity was noted following the larva-pupa ecdysis, a trend that was not observed in the treatment groups (Figure 3A). Spectrophotometric analysis of total protein levels in the control group indicated a gradual increase from day 8 of the final larval instar to 24 hours post-pupal stage. Although a gradual increase in protein levels was recorded from day 8 to day 12 in the treatment groups, these levels remained significantly lower than those in the control group (Figure 3B). The impact of chloroquine on the relative abundance of major hemolymph proteins in the fat body was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3C). Within the circulatory system of insects, a predominant class of lipoproteins known as lipophorins is responsible for the transport of diacylglycerol. In the control group, lipophorin levels, which were initially low on day 8, exhibited a rapid increase, peaking at the pharate pupal stage before declining at 24 hours post-pupal stage (Figure 3D). In Groups I and II, lipophorin levels measured on day 8 were significantly higher than those in the control group; however, this trend was reversed by day 10 (Figure 3D). Storage proteins, which serve as reservoirs for amino acids in silkworms, represent another significant group of hemolymph proteins. In the control group, the relative density of storage proteins, which was at average levels on day 8, demonstrated a slight increase from the pupal stage onward (Figure 3E). Conversely, the levels of storage proteins in Groups I and II were significantly lower on days 8 and 10 compared to the control group (Figure 3E). Notably, on the final day of the experiment, Group II exhibited significantly higher levels of storage proteins compared to the control group (Figure 3E). The 30K proteins, which belong to a family of multifunctional proteins, are abundant in *Bombyx mori* hemolymph. In the control group, the highest relative abundance of these proteins was observed on day 8, followed by a decline from the pharate pupal stage onward (Figure 3F). In Group I, the relative abundance of 30K proteins was significantly lower than that observed in the control group on day 8, while no significant change was detected in Group II on the same day. However, Group II demonstrated a significant increase in the relative abundance of 30K proteins compared to the control group on day 10; nonetheless, a significant decrease was recorded in both treatment groups on day 12 (Figure 3F).

Lysosomal activity was evaluated through Lysotracker staining (Figure 4A) and the measurement of acid phosphatase activity (Figure 4B). In the control group, the highest levels of Lysotracker-positive staining were recorded on day 8 of the final larval stage, followed by a gradual decline until the conclusion of the experiment (Figure 4A). In Group I, the initially weak Lysotracker staining observed on days 8 and 10 exhibited an increase by day 12. Conversely, Group II displayed weak Lysotracker staining consistently across all examined days (Figure 4A). Acid phosphatase activity reached its peak on day 8 in the control group, subsequently decreasing until the 24-hour pupal stage (Figure 4B). In Group I, enzyme activity was significantly lower than that of the control group on days 8 and 10 but demonstrated a notable increase by day 12. In Group II, acid phosphatase activity remained significantly lower in comparison to the control group and exhibited a decline from day 8 to day 12 (Figure 4B).

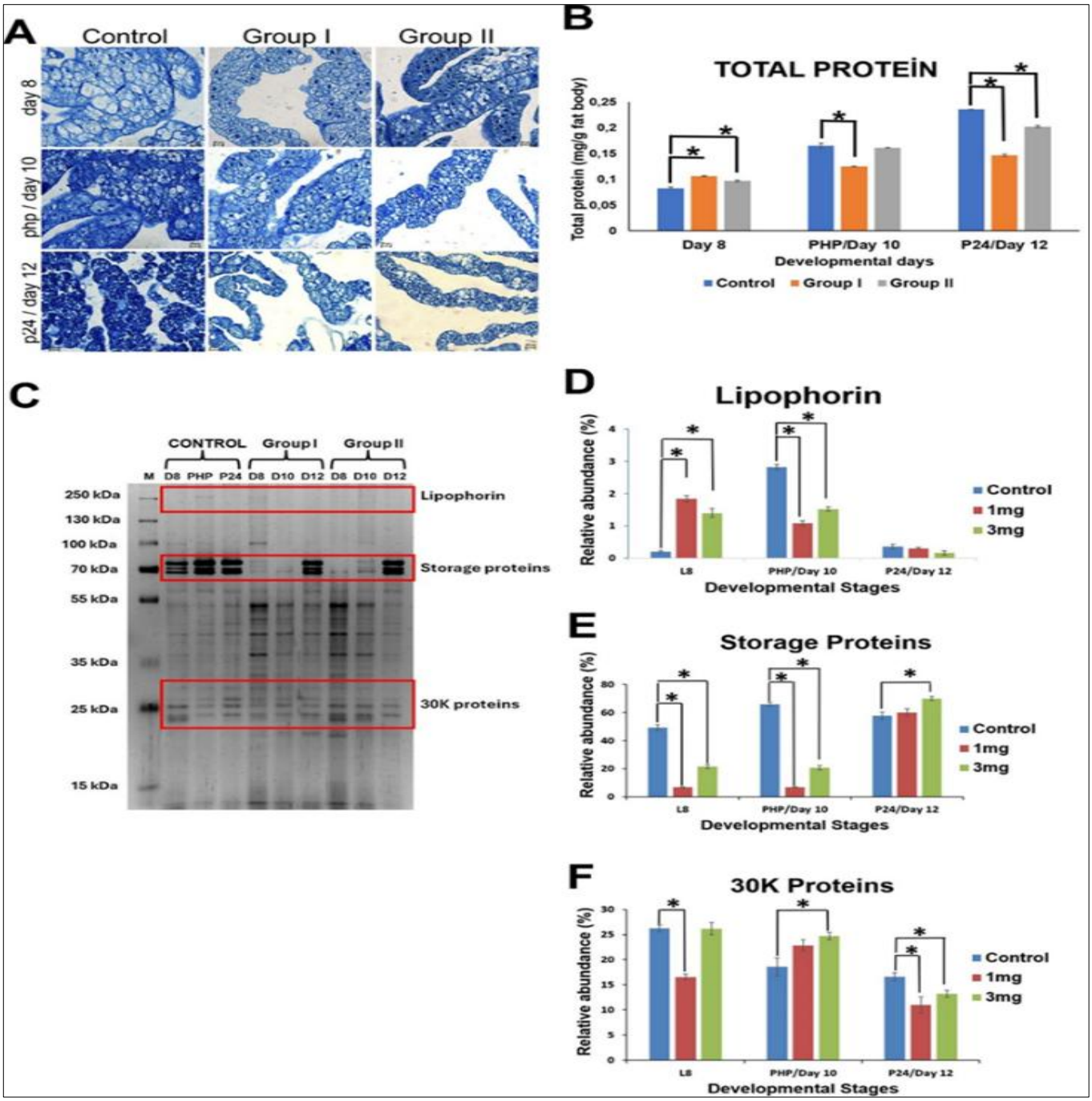

Fig 3: The protein content in the fat bodies of both the control and treatment groups was histochemically assessed using mercury bromophenol blue staining (A), and total protein amounts were evaluated spectrophotometrically (B). Protein profiles in the fat bodies of both control and treatment groups were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (C). The relative abundance of protein bands corresponding to major hemolymph proteins, including lipophorin, storage proteins, and 30K proteins, were quantified using Chemidoc MP software. In the gel photograph, the marker (M) is shown alongside the day 8 of the 5th larval stage (D8), the pharate pupal stage (PHP), and the 24th hour of the pupal stage (P24) in the control group. In Groups I and II, day 8 (D8), day 10 (D10), and day 12 (D12) of the 5th larval stage are represented. The application of chloroquine initially resulted in an increase in the relative density of lipophorin, which was subsequently followed by a decrease (D). A notable reduction in storage protein levels was evident following chloroquine administration (E), and a discernible impact was observed on the relative abundance of 30K proteins. Mean ± standard deviation is indicated on the bars, and significant differences are marked with an asterisk ($p \leq 0.05$).

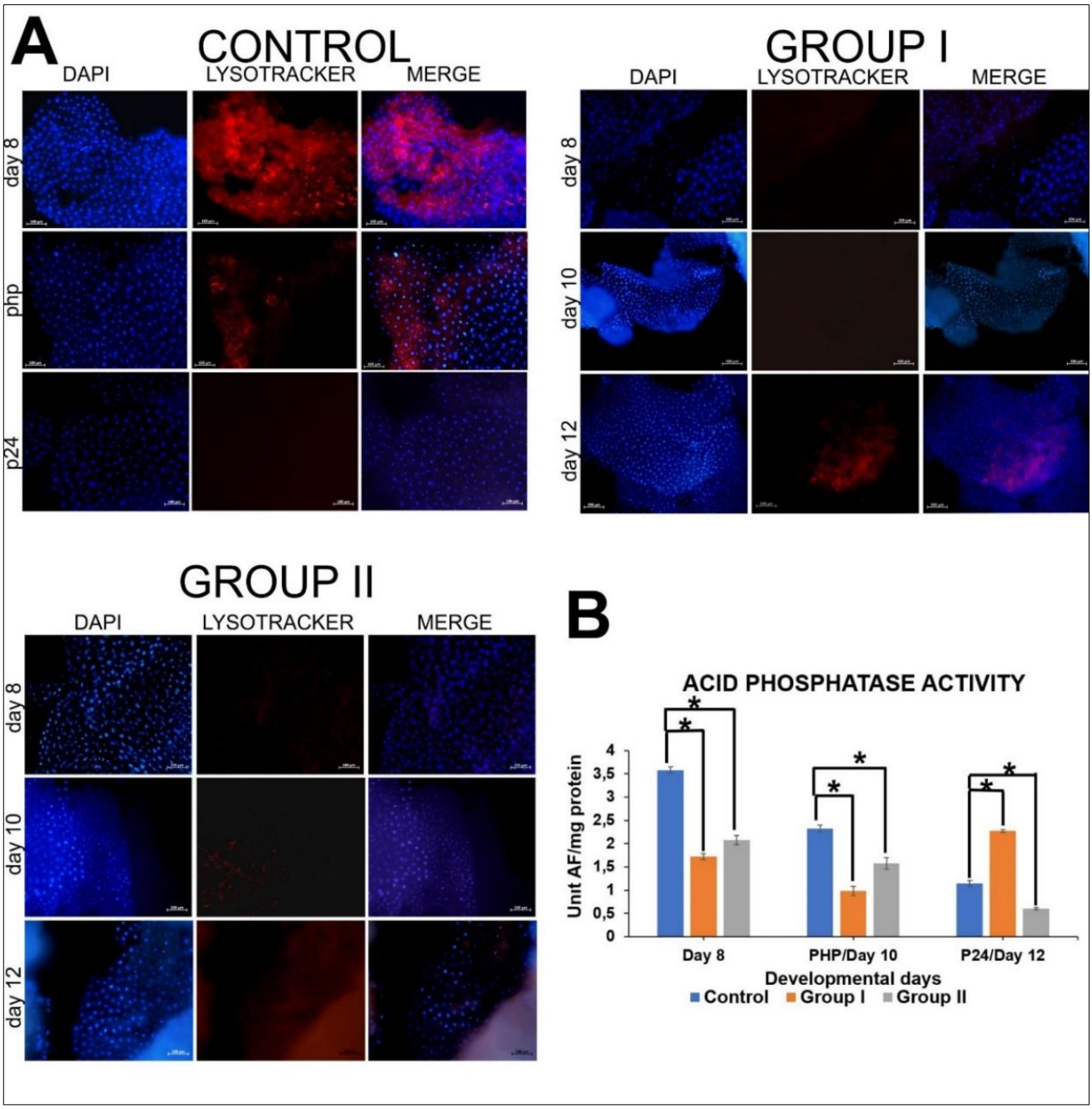

Fig 4: Lysosomal activity in the fat bodies of both the control and treatment groups was assessed using Lysotracker staining (A) and spectrophotometric measurement of acid phosphatase activity (B). Day 8 of the 5th larval instar (day8), pharate pupal stage in the control group (PHP/ day 10 of the 5th instar in the treatment groups (day10), 24 hours of the pupal stage (P24) in the control group/day 12 of the 5th larval stage (day 12) in the treatment groups. Mean ± standard deviation is indicated on the bars, and significant differences are marked with an asterisk ($p \leq 0.05$).

Discussion

This research elucidates the disruptive impacts of chloroquine-induced autophagy inhibition on the metabolic processes of the silkworm fat body, with particular emphasis on its principal constituents. Autophagy is essential for maintaining metabolic homeostasis, particularly by regulating energy metabolism through the degradation of lipid droplets and soluble cytosolic proteins within lysosomes (Arias & Cuervo, 2011; Kim & Lee, 2014) [1, 23]. During metamorphosis in holometabolous insects, energy balance is typically negative (Downer, 1981) [12] due to substantial energy expenditure without external energy sources. Consequently, the fat body's role in mobilizing energy reserves accumulated during the larval stages becomes increasingly significant (Downer & Matthews, 1976) [13]. In the control group larvae, the prepupal stage began on day 7 of the fifth larval stage following gut purge and persisted for a duration of three days. Larval-pupal ecdysis occurred on day 11. However, following chloroquine injections, while cocoon spinning was noted, larval-pupal ecdysis was significantly inhibited. The fact that silencing of autophagy-related (Atg) genes in *Bombyx mori* results in increased mortality at prepupal and pupal stages also emphasizes the essential function of autophagy during metamorphosis (Tian et al., 2013) [42]. During the life cycle of holometabolous insects, autophagy plays a crucial role not only in the efficient utilization of nutrients derived from degenerating larval tissues but also in the development and differentiation of new adult structures (Ryoo & Baehrecke, 2010) [36]. The inhibition of remodeling processes observed in chloroquine-treated groups, along with the preservation of larval characteristics, may be associated

with the function of autophagy in fat body remodeling. The involvement of autophagy in the regulation of adipocyte differentiation has been demonstrated across various models. For instance, Singh et al. (2009) [39] reported that the inhibition of autophagy through RNA interference impeded the differentiation of precursor adipocytes into mature adipocytes. The significant inhibition of larval-pupal ecdysis and fat body remodeling observed following chloroquine treatment can be attributed to disturbed energy metabolism in the fat body, likely due to the inhibition of autophagy. These findings underscore the critical importance of autophagy in fat body during developmental processes.

Lysosomal acidification and elevated enzyme activities are key indicators of autophagy (He & Klionsky, 2009) [17]. Notably, acid phosphatase has been associated with various metabolic processes. For example, Poiani and Cruz-Landim (2012) [34] demonstrated that acid phosphatase is involved in lipid, protein, and glycogen metabolism during the metamorphosis of *Apis mellifera*. Furthermore, the enzyme's involvement in high-energy metabolic processes, as well as in growth, development, maturation, and histolysis, has been well-documented (Pant & Morris, 1972) [32]. In our study, the elevated level of acid phosphatase observed in the fat body of the control group during the prepupal stage aligns with the high energy demands characteristic of this developmental stage. This finding indicated a possible involvement of autophagy during this period. Furthermore, the significantly reduced enzyme activities observed in the treatment groups provided evidence that chloroquine-induced lysosomal dysfunction, as reported by de Duve et al. (1974) [14], occurred.

To examine the impact of chloroquine-induced autophagy inhibition on the fundamental components of fat body, we conducted an analysis of protein profiles as well as alterations in total protein, carbohydrate, and lipid levels. Our study confirmed the accumulation of proteins in the fat body beginning at the pharate pupal stage in the control group, revealing a negative correlation between acid phosphatase activity and protein accumulation. Previous research conducted on the fat body of *Calliphora* indicated that acid phosphatase causes the degradation of protein granules stored within cells (de Priester et al., 1979) [9]. The findings of our study, in conjunction with existing literature, suggested that a reduction in acid phosphatase activity is essential for the realization of protein storage in fat body, which is critical for subsequent developmental processes. Consequently, the elevated protein levels observed on day 8 in the chloroquine-treated groups attributed to diminished acid phosphatase activity. In the investigations conducted on mouse liver and cardiac cells have demonstrated that chloroquine promotes the accumulation of autophagosomes and inhibits proteolysis within proteasomes (Doeppner et al., 2022) [11]. Similarly, Lenz and Holzer, (1984) [28] reported that chloroquine inhibits vacuolar proteinase activities in *Saccharomyces cerevisiae*. Nevertheless, despite the low enzyme activity observed at later stages in treatment groups, the reduced protein levels indicated that factors beyond acid phosphatase activity in fat body, such as reduced recovery of amino acids and proteins as a result of inhibition of degeneration in larval tissues, which is known to be mediated by excessive autophagy.

Chloroquine treatment resulted in significant alterations not only in the total protein quantities of fat bodies but also in the protein profiles. Given the role of lipophorin as a reusable non-internalised shuttle without destruction (Chino, 1985; Tsuchida and Wells, 1988) [7, 43], alterations in the relative abundance of the lipophorin protein following chloroquine treatment in the insect hemolymph may be related to the rate of its synthesis in fat body. Insect storage proteins, which are another important class of hemolymph proteins, synthesized in the larval fat body and subsequently released into the hemolymph are selectively reabsorbed by the fat body during the larval-pupal metamorphosis, resulting in the formation of dense protein particles within the cells (Han et al., 2017) [15]. Previous studies have shown that the uptake of storage proteins occurs via endocytosis and that this process is selectively mediated by specific receptors (Haunerland, 1996) [16]. Autophagy and endocytic pathways cooperate at some stages and share many components of molecular machinery. In studies on the impact of chloroquine on endocytosis, it has been observed that chloroquine exerts an inhibitory effect on endosomal function by disrupting receptor-ligand interactions (Korelenko et al., 1992) [25]. Therefore, the decrease observed in the relative abundance of storage proteins in the fat body following chloroquine treatment in this study may be attributed to the inhibitory effects of chloroquine on endocytosis. Another prominent group of proteins found in hemolymph are the 30K proteins. It was observed that the concentrations of 30K proteins, which is synthesized by the fat body in a stage-specific manner and subsequently secreted into the hemolymph (Izumi et al., 1981) [19], began to increase on second half of the fifth larval stage (Uranli et al., 2010) [45]. The subsequent decline in the relative abundance of these proteins in the fat body of control group from day 8 onwards may be attributed to a significant transfer of proteins into the hemolymph. Furthermore, the marked decrease in 30K proteins following chloroquine treatment may be associated with changes in the tissue's synthesis capacity or the rate of protein release from the fat body into the hemolymph.

In relation to glycogen, which is synthesized from UDP-glucose derived from dietary carbohydrates or amino acids, our findings indicated that glycogen levels decreased from the prepupal to pupal stages in the control group, corroborating previous studies (Pant & Morris, 1972) [32]. Chloroquine treatment did not result in a significant alteration of glycogen levels when compared to the control group; however, spectrophotometric measurements demonstrated an increase in sugar content within the fat body, suggesting an enhanced uptake of sugar into the tissue. Cynober et al. (1987) [8] reported that chloroquine facilitates insulin-mediated sugar uptake in chick embryo fibroblasts. The findings of this study align with previous research, indicating that chloroquine influences glucose metabolism; but the specific mechanisms underlying this effect require further investigation.

The role of the fat body as the primary lipid storage organ in insects is essential for maintaining normal physiological processes (Park et al., 2013) [33]. Lipid catabolism is carried out by the coordinated activation of lipophagy, defined as the selective degradation of lipids, and lipolysis (Schulze et al., 2017) [38]. In the control group, the increased activity of acid phosphatase during the prepupal stage, along with a reduction in lipid levels from the pharate pupal stage, indicated lipid utilization through lipophagy. In contrast, the significantly elevated lipid levels and reduced enzyme activities observed on

days 8 and 10 in group I suggested an inhibition of lipophagy. This finding aligns with previous observations of intracellular lipid accumulation in HepG2 cells subjected to chloroquine treatment (Xu et al., 2022) [50]. Nevertheless, the absence of significant differences in lipid levels in Group II, despite increasing doses of chloroquine, suggested the presence of complex regulatory mechanisms governing lipid metabolism that needs further investigation.

Conclusion

Our research has demonstrated that chloroquine-mediated inhibition of autophagy results in significant alterations in the metabolic functions of the fat body. These alterations highlight the critical role of autophagy in the metabolic processes of the fat body that occur during larval-pupal metamorphosis. Furthermore, our findings raise new questions that necessitate further investigation; addressing these inquiries will enhance our understanding of the role of autophagy in regulating insect metabolism. The insights gained may provide new perspectives for improving the productivity of economically significant insect species, such as silkworms, and for developing innovative strategies for the control of insect pests.

Acknowledgments

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