Vol. 2 | Issue 1
December 2023

Journal of Biomedical Research and Therapeutics

Hen's egg yolk plasma as a source of proteins with therapeutic potential

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Abstract: The primary biological function of avian eggs is giving rise to new life. Consequently, they contain essential elements for developing embryos, and numerous egg compounds, including proteins and peptides possess biological activity. For this reason, these substances have great potential to be used in the prevention and treatment of many diseases. This review summarizes available literature regarding the biological activity of egg yolk plasma proteins including Low-density Lipoproteins, Immunoglobulin Y, and newly discovered polypeptide complex- yolkin.

Keywords: Immunoglobulin Y (IgY), yolkin, hen egg yolk, immunoregulatory activity, natural substances with therapeutic potency

Submitted: 13 October 2023, revised: 20 February 2024, accepted: 21 February 2024

1. Introduction

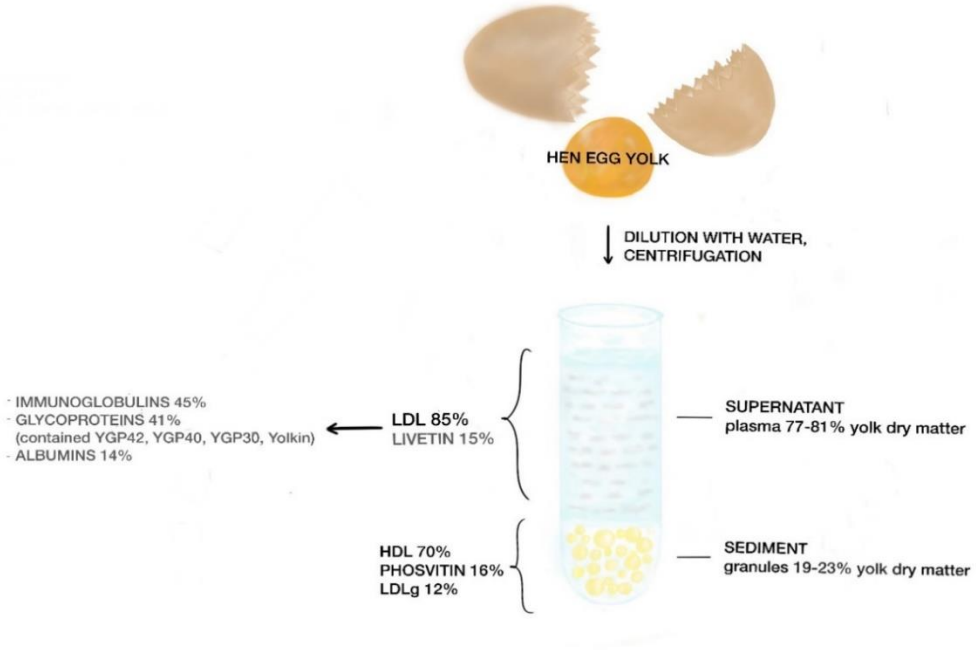
There is no greater creation of nature than an egg. As a reproductive cell, it ensures the development of the embryo, regardless of the laying hen. Yolk is of particular importance as it contains not only nutritionally high-value proteins, lipids and minerals but also several regulatory and defense protein factors to ensure the proper development and protection against various infections [Mine, 2007; Réhault-Godbert et al., 2019; Meng et al., 2019; Zhang et al., 2021; Arena & Scaloni, 2018]. For example, egg yolk is a source of substances exhibiting antibacterial, anti-cancer, anti-inflammatory, antihypertensive (angiotensin-converting enzyme (ACE) inhibition), antidiabetic or anti-neurodegenerative activities [Zhang et al., 2021; Arena & Scaloni, 2018; Zambrowicz et al. 2015a, b; Kazana et al., 2023]. These egg yolk-derived substances, essential for the bird embryo, can also be of great importance for humans and find application as natural drugs or nutraceuticals. Although most protein substances from each part of the egg are currently described, substances contained in the yolk plasma appear to be insufficiently characterized. Therefore, the review describes biologically active proteins originating from hen egg yolk plasma and presents the potential of their therapeutic role.

2. Composition of egg yolk

Yolk makes up approximately 36% of a hen egg. The dry matter of the yolk reached the value of ~50% and contains lipids (62.5%), proteins (33.0%), carbohydrates (1.2%), and minerals (3.5%) [Anton, 2007]. Most egg yolk substances are synthesized in the liver, then transported by the blood to the ovary, and incorporated into the egg cell through

receptor-mediated endocytosis [Meng et al., 2019]. Egg yolk can be easily separated into main fractions called plasma and granules after water dilution and centrifugation [Leśnierowski and Stangierski, 2018]. Yolk proteins are also divided into granule proteins and plasma proteins (Fig. 1). The granules contain α- lipovitellins, β- lipovitellins, high-density lipoproteins (HDLs), phosvitin, and low-density lipoproteins (LDLs) (15% of total content in yolk). The main proteins in plasma are LDL (85% of the total content of yolk) and livetin [Zambrowicz et al., 2014; Wang et al., 2018]. Livetin is a heterogeneous fraction and is composed of: α-; β- , and ɤ-livetin. The main component of α-livetin is albumin, which accounts for 14% of the proportion of all proteins in the entire livetin fraction whereas α-2-glycoprotein (41%), YGP42 and YGP40 are the main components of β-livetin. Also present in β -livetin is YGP30 showing 75% homology to YGP40. Immunoglobulin Y (IgY) accounts for 41% of the total livetin and is the main component of gamma livetin [Jolivet et al., 2008; Zambrowicz et al., 2023]. Egg yolk plasma also contains a newly discovered polypeptide complex accompanying IgY named yolkin [Polanowski et al., 2012, 2013].

Figure 1. Separation of hen egg yolk into granules and plasma fractions.



3. Low-density Lipoproteins (Lipovitellenin) (LDL)

Low-density lipoproteins, the main constituents of yolk (about 2/3 of the total dry matter) are synthesized in the liver of the laying hen and transported with the blood to the ovary and transferred to the yolk during egg formation [Anton, 2007]. Apolipoprotein B in hen blood gives rise to different apoproteins in LDL [Jolivet et al., 2006; 2008]. LDLs are composed of 83-89 % lipids (26% of them are phospholipids) and 11-17% proteins [Anton, 2007; Wang et al., 2018]. LDLs consist of two subgroups differing in size: LDL1($10x10^6$Da) and LDL2 (3×10^6 Da), both LDL1 and LDL2 represent 80% of LDLs [Anton et al., 2003; Anton, 2007; 2013]. Six main apoproteins are included in LDL. The predominant group of apoproteins reaching a 70% share in LDL has a molecular weight of 130 kDa. The second group is made up of apoproteins with a molecular weight of 15 kDa, and their share in LDL is 20%. Four other apoproteins with molecular masses between 55 and 80 kDa are minor constituents. These apoproteins are highly hydrophobic and flexible molecules (40% of the amino acids of LDL apoproteins are hydrophobic) [Anton et al., 2003; Anton, 2007].

LDLs possessed an antibacterial effect against pathogenic Streptococcus strains such as *S. mutans* and *S. sanguis*. LDL activity towards those gastrointestinal pathogens drastically increases after stimulated digestion [Brady et al. 2006]. It

suggests, that potential LDLs-based anti-inflammatory drugs administered orally can be very effective. Vitelenin, which is one of the LDL apoproteins of egg yolk plasma can also be a precursor of biologically active peptides named glycopeptide A and glycopeptide B. The biological effect of those peptides can result from the presence of sialic acid in their structure. Sialic acid is a naturally occurring carbohydrate, which parcipitates in blood protein half-life regulation, a variety of toxin neutralization, regulation of cellular adhesion, and inhibition of cell cytolysis [Abdou et al., 2013]. According to Abdou et al. [2013] those peptides are good carriers of sialic acid and increase its bioavailability.

There is little data available regarding the therapeutic effect of LDLs, however, they are used in veterinary medicine as a protective substance used in the cryopreservation of spermatozoa. The addition of LDLs improves the fertilization potential of sperm, improves spermatoza motility, reduces Reactive Oxygen Species (ROS) impact on semen [Souza et al., 2015; Prapaiwan et al., 2016; Dong et al., 2011].

4. Immunoglobulin Y (IgY) (γ livetin)

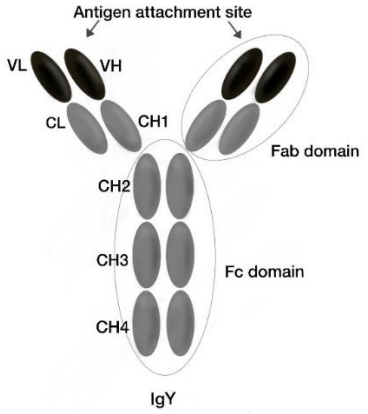
The 3 main fractions of livetin are α, β, and γ livetin. A major fraction of γ livetin is immunoglobulin Y (IgY) [Wang et al., 2018].

IgY is the main class of antibody present in avian blood responsible for immune response functions. IgY is produced by plasma cells beginning 5 day following immunization of laying hens [Schade & Chacana, 2007]. IgY is involved in opsonization, complement system activation, and most effector functions in the chicken [Karlsson et al., 2004; Lee et al., 2021]. IgY is also responsible for the passive immunity of the hatched chicken until its own immune system reaches full capacity. IgY is transferred from the maternal bloodstream to the egg yolk, mediated by oocyte membrane receptors, and transferred by special receptors from the embryonic yolk sac to the embryonic bloodstream [Schade & Chacana, 2007].

4.1. Physicochemical characteristics of IgY

IgY concentration is estimated to be 100-200 mg per egg and varies significantly among individuals [Schade & Chacana, 2007]. The pI of that glycoprotein is in the range of 5.7-7.6 [Sun et al., 2001; Leiva et al., 2020]. IgY is stable at temperatures ranging between 30°C and 70°C and at a wide range of pH values from 3.5 to 11.0 [Leiva et al., 2020]. The molecular structure of the IgY molecule consists of two heavy chains and two light chains, each containing constant and variable regions (Fig. 2). The variable regions in the molecule are critical for antigen recognition. The constant regions provide the effector function [Rahman et al., 2013; Lee et al., 2021].

Figure 2. The general structure of Immunoglobulin Y. Molecule containing two heavy and two light chains. The heavy chain consists of a variable domain (VH) and constant domains (CH1, CH2, CH3, and CH4). The light chain has one variable domain (VL) and one constant domain (CL). The fragment antibody (Fab) domain binds to antigenic epitope. The fragment crystallizable (Fc) domain of the molecule has biological effector functions (Rahman et al., 2013).

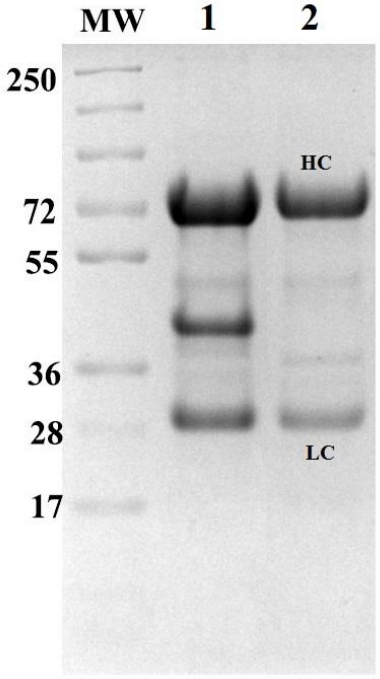


The molecular weight (MW) of the native molecule is 180 kDa, however, the MW of IgY was validated by mass spectrometry and found to be 167.250 Da [Sun et al., 2001; Lee et al., 2021]. SDS- Polyacrylamide gel electrophoresis gave the possibility to analyze protein under denaturing conditions, where IgY molecule is separated into two heavy and two light chains (Figure 3). Each heavy chain and light chain has a MW ~ 70 kDa and ~ 25 kDa, respectively [Leiva et al., 2020].

4.2. IgY for therapeutical or prophylactic application

Due to its properties, IgY has been used in a variety of research fields and industry sectors such as immunoassays, immunochemistry, clinical chemistry, and medicine. IgY-based immunoassays are used to measure the concentration of proteins via ELISA diagnostic test, while in immunochemistry IgY is used for the detection of antigens of different origins, and the contamination of food with toxins or drugs. Furthermore, IgY has been used for the diagnosis, prevention, and treatment of infections caused by bacteria and viruses, and has also been used as a tool in proteomics and in functional food [Schade & Chacana, 2007; Shade et al., 2005; 2007; da Silva and Tambourgi, 2010; Lee et al., 2021].

Figure 3. SDS Polyacrylamide gel electrophoresis (12% gel) of IgY purified according to the method described by Polanowski et al., 2013. MW – molecular weight marker (250- 17 kDa); 1- protein fraction of yolk plasma obtained after saturation with ammonium sulfate (0.4), 2- IgY isolated from saturated proteins (1) on size exclusion chromatography on sephacryl S100 HR resin. LC- light chain of IgY, HC heavy chain of IgY.



IgY is a highly conserved homolog of human immunoglobulin G (IgG) that has shown benefits in the treatment and prevention of human infectious diseases and has a favorable safety profile [Sun et al., 2001; Lee et al., 2021]. Additionally, compared with mammalian IgG, chicken IgY has 3 to 5 times more affinity and reacts more rapidly to the same antigens when tested in competition assays. Specific IgY antibodies can be easily obtained by immunizing the hen with the antigen of interest [Raham et al., 2013]. IgY can be effectively used in the treatment of intestinal infections in children. It has been shown that specific IgY against *Salmonella* antigens can inhibit the adhesion of this bacteria to epithelial cells [Lee et al., 2002]. Also, the oral administration of the IgY antibody resulted in a protective effect toward human rotavirus [Sarker et al., 2001]. The results of recent clinical trials involving 342 children proved that IgY can reduce the duration of diarrhea in children with acute non-bloody diarrhea [Gaensbauer et al., 2017].

IgY can be a potential drug for the treatment of colitis because demonstrates a protective effect against tumor necrosis factor (TNF) which is implicated in the pathogenesis of this disease [Worledge et al., 2000; Lee et al., 2021]. Sunwoo and Sim [2004] have proven the possibility of obtaining IgY antibodies against dietary gluten proteins, therefore this protein may play a positive role in the autoimmune disorder of celiac disease.

Gastritis, peptic ulcers, and gastric adenocarcinomas are associated with the gram-negative bacteria *Helicobacter pylori* [Leiva et al., 2020]. IgY is of great importance for humans because it also has a protective effect against *Helicobacter pylori* [Lee et al., 2021; Kovacs-Nolan & Mine, 2012]. Specific IgY antibodies against the immunodominant protein of *H. pylori* can be an effective tool in the treatment of infection caused by those bacteria [Shin et al., 2003]. In turn, the protective effect of IgY against *Pseudominas aeruginosa* makes this protein useful in the therapy of patients suffering from cystic fibrosis. This disease leads to secondary infections in the respiratory tract caused by numerous bacteria species including *P. aeruginosa.* Orally administered IgY was able to reduce chronic infections in cystic fibrosis patients [Kollberg et al., 2003; Schade et al., 2007].

IgY is of great significance in another sector of medicine such as dentistry and plays a protective role in dental caries periodontitis and gingivitis [Hatta et al., 1997; Schade et al., 2005; 2007; Rahman et al., 2013]; Oral treatment with anti-*Streotococcus Mutans* IgY effectively inhibited *S. mutans* adherence to saliva-coated hydroxyapatite discs [Hatta et al., 1997]. In 2014, Nishihara et al.[2014] carried out a clinical trial to evaluate the efficacy of different tablets containing IgY against *S. mutans* glucosyltransferase, versus treatment with probiotic Lactobacillus or xylitol on caries risk. They found that the levels of *S. mutans* decreased in patients treated with IgY or probiotic but not in patients treated only with xylitol. Additional results obtained from other clinical trials confirmed the reduction of *Porphyromonas. Gingivalis* and gingival bleeding index when IgY was applied to the teeth of patients [Nguyen et al., 2017].

The coronavirus disease epidemic that began in 2019, recognized by WHO in 2020 as a pandemic, forced the search for efficient intervention strategies. Therefore, recent research on IgY has mainly focused on its use in the treatment of SARS-CoV-2. Yeh et al. [2022] analyzed the efficiency of IgY antibody specific toward S1, S1 receptor-binding domain (S1-RBD), or S2 subunits of the SARS-CoV-2 spike (S) protein on an animal model with the use of Syrian hamsters. They have proven that IgYs were immunoreactive against S1, S1-RBD, and S2 subunits. In their study, with Syrian hamsters, the combination of IgYs for S1-RBD and S2 subunits administered before or after SARS-CoV-2 infection effectively restored body weight loss and reduced intrapulmonary lesions and the amount of

immunoreactive N protein-positive cells, which were caused by SARS-CoV-2 infection. According to Lee et al. [2021], the potential anti-SARS CoV-2 effect of specific IgY may be considered through a few mechanisms such as neutralization or binding of SARS-CoV-2, binding to the spike protein on the surface of the virus, competing with the binding of the viral spike protein to the human angiotensin-converting enzyme 2 receptor to prevent cell entry and infection. Additionally, anti-SARS-CoV-2 IgY may agglutinate SARS-CoV-2 on the surface of the mucosa, preventing entry across the mucosa.

The advantages of IgY stem from its unique traits as well as the stability of molecule in the oro-gastrointestinal tract and safety profile [Rahman et al., 2013; Lee et al., 2021]. Furthermore, IgY is easy to produce, and low cost because it can readily be generated in large quantities, and an extraordinary amount of IgY antibody produced by one hen in one year reached between 20g and 40g IgY in total [Lee et al., 2021; Shade et al., 2005]. Moreover, IgY technology can be easily sampled by a non-invasive method (with reduced painful manipulations) based on the laying hen's immunization, collection of eggs, and IgY isolation from egg yolk [Lee et al., 2021]. Furthermore, IgY antibodies are particularly resistant to low pH, high temperatures, and proteolysis. IgY can be effectively administered prophylactically and therapeutically to detect and neutralize pathogens without activating the host's native immune system [Lee et al., 2021]. The IgY antibodies can be used in different forms, those for oral administration, such as table eggs, liquid and powdered eggs, encapsulated nutraceuticals, and drugs [Schade et al., 2005; 2007], IgY has been incorporated into toothpaste and mouthwash to reduce dental plaque in humans. Furthermore, IgY may be an easily accessible nasal therapeutic agent for rapid prophylaxis and therapeutic development towards a variety of pathogens. In particular, passive immunoprophylaxis by surface treatment with IgY e.g. via nasal spray [Lee et al., 2021]. Numerous IgY-based drugs have already entered the market, including Helicobacter pylori NCT02721355 and Pseudomonas aeruginosa NCT01455675 [Leiva et al., 2020].

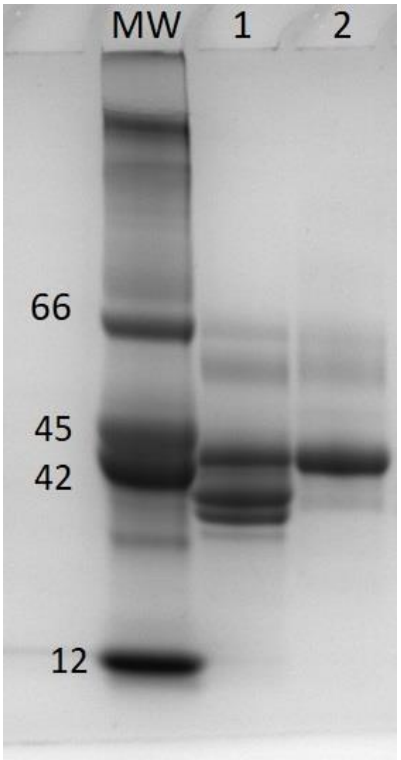
5. Yolkin and Yolk Glycopeptide 40- (YGP40)

Hen egg yolks are an excellent material for manufacturing nutraceuticals and pharmacological preparations [Leśnierowski and Stangierski, 2018 Leiva et al., 2020]. One particularly interesting compound is yolkin [Polanowski et al., 2012; 2013; Kazana et al., 2023, Zambrowicz et al., 2023].

5.1. Physicochemical characteristics of yolkin

Yolkin is a mixture accompanying chicken IgY- γ livetin in yolk plasma, consisting of several polypeptides and peptides of different molecular weights (Fig.4). [Polanowski et al., 2013; Zambrowicz et al., 2017, 2018]. For example, the yolkin preparation obtained by Polanowski et al. [2013] was composed of peptides of a molecular weight in a range from 1.0 to 35 kDa. The amino-acid profile of yolkin is characterized by the presence of a great amount of acidic amino acid residues and a low amount of methionine [(Polanowski et al., 2013]. Yolkin polypeptides are products of limited cleavage of vitellogenin II by cathepsin D, occurring during formation of the egg [Yamamura et al., 1995]. The fraction of molecular weight (MW) about 3700 kDa is free of carbohydrates and starts at position 1732 in the vitellogenin II amino acid sequence, while the fractions with MW between 15600 - 36000 Da are glycoproteins corresponding to the amino acid sequence of vitellogenin II starting at position 1572 [Polanowski et al., 2013; Zambrowicz et al., 2018].

Figure 4. SDS Polyacrylamide gel electrophoresis (12% gel) of plasma-derived proteins with potential therapeutic effect. MW – molecular weight marker (bovine serum albumin (66-70 kDa, ovalbumin 45 and 42 kDa and cytochrome c 12 kDa); 1-yolkin isolated from egg yolk according to Polanowski et al., (2013). 2-Yolk glycopeptide 40 (YGP-40).



The results of research on yolkin carried out in recent years in research centers in Wroclaw (Poland) prove that yolkin is a potential therapeutic compound. As it is a recently discovered compound, unlike IgY, the research is preliminary and now includes *in vitro* and *in vivo* tests on animals.

5.2. Immunoregulatory activity

The recently obtained results demonstrate that yolkin possesses immunoregulatory activity [Polanowski et al., 2012; 2013; Zambrowicz et al., 2017; Kazana et al., 2020, 2022a. Obmińska-Mrukowicz et al., 2021]. However, the mechanisms of action of yolkin are still under investigation. Immunomodulatory properties of yolkin were confirmed in the human whole blood cultures which mimic function of the immune system *in vivo* and in the selected cell lines such as mouse bone marrow-derived macrophages of the BMDM cell line, and murine macrophage-like cell line J774.2 [Polanowski et al., 2012; 2013; Zambrowicz et al., 2017; Kazana et al., 2020, 2022a]. It has been shown that yolkin used as an exogenous agent can induce the secretion of key factors for the functioning of the immune system such as interferons α/β (IFNs α/β), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), chemotactic interleukin 8 (IL-8), tumor necrosis factor-alpha (TNF-α) and interleukin 10 (IL-10) [Polanowski et al., 2012, 2013; Zabłocka et al., 2014, 2021; Kazana et al., 2020, 2022a].

Obmińska-Mrukowicz and co-workers [2021] confirmed the immunomodulatory activity of yolkin with the results of studies conducted on mice. They proved that yolkin influences the development of the immune response and also the phenotype of cells in lymphoid organs. It was shown that yolkin can participate in the induction of maturation and stimulatory signals in immature two main types of lymphocytes: T cells and B cells. Treating mice with yolkin diminished the percentage of double-positive cells and caused the growth of the content of single-positive CD4+ and CD8+ cells in the thymus and the level of CD19+B cells in the spleen and mesenteric lymph nodes. T cells were affected by yolkin as evidenced by a significant increase of mature thymocyte subset, stimulation of mitogen-induced proliferation of thymocytes, and activation of MAP kinases in Jurkat cells [Obmińska-Mrukowicz et al., 2021].

The immunoregulatory properties of yolkin may also result from its effect on the secretion of nitric oxide, which is an important molecule exerting beneficial results as an antibacterial, antiparasitic, antitumor, and antiviral agent [Zabłocka et al., 2014; Kazana et al., 2020, 2022a, Zambrowicz et al., 2023]. Yolkin can increase iNOS (inducible Nitric Oxide Synthase) expression and can upregulate NO production in BMDM cells [Kazana et al., 2020].

Recently, Kazana et al. [2020, 2022a] examined the impact of yolkin on the maturation and function of macrophages using as a model mouse bone marrow-derived macrophages of the BMDM cell line. Macrophages are key immune cells participating in the immune response to pathogens and tumors, maintaining tissue homeostasis, promoting the repair processes, and controlling immune response in numerous diseases [Shapouri-Moghaddam et al., 2018]. The studies show that yolkin can regulate macrophage profiles [Zabłocka et al., 2014; Kazana et al., 2020, 2022]. Kazana et al., [2022a] proved that yolkin down-regulates BMDM cell proliferation and simultaneously increases the cell surface marker expression of CD80/CD86, which indicated macrophage polarization towards the macrophages M1 phenotype.

The stimulating effect of yolkin on immune system cells may also be related to the ability of yolkin to upregulate the phosphorylation of extracellular signal-regulated kinase 1/2(ERK1/2), c-Jun N-terminal kinase (JNK), and PI3K/Akt kinases, which provided an increased mRNA expression and production of cytokines TNF-α, IL-6, and IL-10, in BMDM cells [Kazana et al., 2020].

5.3. Neuroprotective activity of yolkin

Due to the fact that yolkin may participate in the regulation of the immune response, it is highly probable that it may also be an important inhibitory factor for the progression of neurodegenerative diseases. In 2016, Lemieszewska et al. proved the neuroprotective properties of yolkin in a rat model of age-related cognitive dysfunction. Aged rats were shown to have reduced behavioral symptoms of aging and improved cognition, learning, and memory [Lemieszewska et al., 2016]. That study proved that yolkin may be a promising candidate for the prevention and treatment of aging-related neurodegenerative disorders. The molecular mechanism of the neuroprotective activity of yolkin is still under investigation.

The level of neurotrophic factors could be associated with the pathogenesis of neurodegenerative disorders [Scalzo et al., 2010; Sopova et al., 2014]. Neurotrophins play a relevant role in neuron cell physiology. One of those neurotrophins is a brain-derived neurotrophic factor (BDNF) involved in the neuron's development, survival, and repair after injury [Scaper, 2008; Bartkowska et al., 2010]. Therefore the mechanisms of the pro-cognitive effect of yolkin may result from its impact on BDNF synthesis and secretion. Firstly, Zambrowicz et al., [2017] showed that yolkin can stimulate whole blood cells to

release significant amounts of BDNF [Zambrowicz et al., 2017)]. In addition, yolkin can upregulate cAMP production and PKA activation in both rat pheochromocytoma PC12 cells and rat hippocampal precursor cells H19-7, resulting in increased CREB factor phosphorylation and activation of BDNF expression [Kazana et al., 2022b, 2023]. Additionally, yolkin can upregulate the expression of carboxypeptidase E/neurotrophic factor α1 (CPE/(NF-α1) [Kazana et al., 2023]. Another possible explanation of the neuroprotective effect of yolkin is its ability to reduce intracellular ROS generation induced by H_2O_2 and modulation of nitric oxide production by nNOS [Zabłocka et al., 2018; Zabłocka et al., 2014].

Due to the above-mentioned biological properties such as regulation of the secretion of neurotrophins, modulation of the immunological system, and support mechanisms of cell protection against ROS, yolkin can be of great importance in the development of new neuroprotective drugs. However additional studies are needed to explain the molecular mechanisms of yolkin neuroprotective activity as well as validation of efficiency in clinical trials.

5.4. Yolk Glycopeptide 40- (YGP40)

Apart from yolkin, the presence of other vitellogenin-derived proteins in egg plasma was confirmed by Yamamura et al. [1995]. They identified a yolk glycoprotein of 40 kDa (YGP40) with an asparagine-linked carbohydrate chain, identified as a C-terminal cysteine-rich fragment of Vt II, the cysteine-rich domain homologous to the D2 region of von Willebrand factor. They also identified a yolk plasma glycoprotein of 42 kDa which was probably the proteolytic product of Vt I [Yamamura et al., 1995]. Recently, Szmyt et al. [2021] produced YGP40 using a recombinant DNA technology and proved that recombinant YGP40 (rYGP40) possesses immunomodulatory activity in test *ex-vivo*. rYGp40 can stimulate human whole blood cells to produce TNF-α and IL-10 and induce NO production. Furthermore, rYGP40 also causes the up-regulation of iNOS expression and NO production in murine bone marrow-derived macrophages (BMDM) [Szmyt et al., 2021].

6. Plasma proteins- precursors of biologically active peptides

Plasma proteins, apart from exhibiting biological activity in their native form, may be precursors of numerous biologically active peptides generated under enzymatic hydrolysis. Arena and Scaloni [2018] using bioinformatic analysis identified 198 peptides associable with putative antihypertensive, antimicrobial, anticancer, antiviral, antibiofilm, anorectic, calcium-binding, and anti-inflammatory activities.

7. Summary

In addition to food consumption, increased attention has been given to exploring the unique biological values of eggs and their applications in the pharmaceutical industry. Hen egg yolks harbor numerous active biological ingredients and have the potential to serve as raw materials in various biomedical industries. In this review, we compiled information about bioactive proteins from the yolk plasma fraction and demonstrated their potential therapeutic importance.

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