

Fascinating Findings from Sensitizing the Wistar Strain Rats Recruited as Peanut-Allergy Model

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Abstract

Whenever feasible, animal-based investigations are accomplished in order to characterize/scrutinize to a nicety, the potential sensitizing-activity of the pre-determined and novel allergenic proteins in suspected food(s) also, for the generation of appropriate humankind therapeutic agents. The main aim of the current study was to confirm the sensitization-operation fulfillment in a Wistar strain model of peanut allergy. 21 out of 42 male Wistar rats, aged 4-6 weeks in the beginning, were randomly subjected to sensitization through a 3-stage protocol, at weekly intermissions, with crude peanut extract.

Subsequently, in proof of the sensitization-phase completion, a variety of proven/endorsed *In vitro* and *In vivo* assessments that represent anaphylactic parameters were evaluated. Eventually, anaphylactic responses of the sensitized Wistar rats were approved by a significant increment in plasma histamine levels and, in anaphylactic-symptom scores [(p = 0.000) and (p = 0.000) respectively, compared to negative controls], as well as, by positive *intra*dermal- and *intra*peritoneal-challenge test outcomes.

In brief, considering the homeostatic similarities between rats and humans, earlier studies have referred to Brown Norway rats as a suitable model for human allergic disorders. But here, putting all together, we certify/testify daringly that the Wistar strain model of peanut allergy resembles the humankind responses of the IgE-mediated food allergies in a near manner.

Keywords: Wistar Strain Model, Peanut Allergy, Anaphylactic Parameters, Adjuvant

Introduction

Adverse immunological reactions to foods are widespread with an acute onset of symptoms/signs following ingestion and typically, mediated by IgE-antibodies [1]. Food specific IgE-antibodies arm the Effector cells; Tissue Mast cells and Blood Basophils, - a condition called 'Sensitization'. Subsequent exposure to the same allergenic food leads to the discharge of a large number of chemical mediators through the effector cells degranulation. Amongst them histamine is assumed as an influential mediator that can induce all the pathological characteristics of allergic disorders [2-4].

Although peanuts (PNs) and tree-nuts originate from different families, however, they have both, been known to contain potent allergens, with a US study reporting PN and tree-nut allergies to specifically, be account for 90% of the IgE-mediated, deadly anaphylactic reactions [5]. Contrary to other food allergies such as Eggs and Cow's Milk, PN allergy is not often outgrown.

Notwithstanding our increased understanding of pathophysiological mechanisms involved in food allergies in recent years, there is still no specific therapeutic/curative option available. Presently, strict avoidance and the prescription of adrenaline, in the event of an accidental exposure, are the extant/residual recommended cares.

Talking of several forms of immunotherapy -being currently under investigations including oral, sublingual, epicutaneous and subcutaneous allergen specific immunotherapies [6,7]- regrettably, the high risk of possible anaphylaxis is a major factor confining the

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development of PN-allergy's immunotherapy in humans [6,8]. On this concern, animal models may play an important role in providing a platform for refining the treatment polices and, ensuring thorough pre-clinical evaluation of their safety, before therapeutic applications in humans.

Definitely, while *In-vitro* and cellular surveys are advantageous for evaluating the allergenicity in food products, however the sensitizing potential as well as, the tolerogenic capacity of foodstuffs can be evaluated merely, via *In-vivo* animal models [9].

To-date, there is no ideal animal model for food allergy (FA). Dogs, pigs, and sheep are typical examples of large animal models that have been utilized in FA studies. Even though the large animals bear significant precedence/preference as models for FA reflecting closely the human-being corresponding allergic entity owing to their physiology and out-bred traits [10,11], however, small animal models are often employed to characterize the underlying immunological pathways.

At a glance, murine models are the best/commonest small animal-models among the rest, with Brown Norway strain being claimed to be appropriate for inducing specific IgE-immunoglobulins after oral-sensitization [12-17]. Of course, other rat-strains have also been evaluated but it is reported that they fail to produce quantifiable levels of antigen-specific IgEs [14].

Concerning the critical interests to know the pathology of the animals being studied and, to understand the impact of the disease-processes on the parameters being measured, the striking characteristics of Wistar-rat as a highly-adaptive alternative model, for comparison polices/purposes, as well as, testing of different therapeutic/interventional procedures can be practically fascinating. Principally, Wistar rats are used as the primary species for ADME (Absorption, Distribution, Metabolism and Excretion) and toxicology studies in early drug-development. In a parallel manner, marked wistar-strain-dependent experimental facilities are extensively available world-wide.

Hence, in an effort to recruit the most relevant rats for FA model, we were prompted to test the Wistar strain model of PN allergy. As a result, the current study was directed, in continuation of our previous study [18], to scrutinize the susceptibility of Wistar rats to PN hypersensitivity following the sensitization/induction protocol with the view of improving/promoting of our understanding as for the temperament of food allergies.

Materials and Methods

Laboratory Animals

A total of 42 male Wistar rats, aged 4-6 week and, weighing 80-120 g at study-start, were obtained from the Animal House of Ahvaz Jundishapur University of Medical Sciences (AJUMS) and shortly, were divided into wiry-cages in colonies of 5 (max). Considering the operating-instruction, the rats were housed in an animal room sustained at $23 \pm 3^{\circ}\text{C}$ and a relative humidity of 30-70% with an altering light-dark cycle of 12h, throughout the research and for at least, one week before the sensitization period for acclimatizing. The animals had free access to PN-free standard rodent-chow and water.

All the processes/handlings involving the investigated wistars were accorded exactly, to Guidelines for the Laboratory Animal Experiments in AJUMS Animal Research and Care Center.

Reagents

The substances/reagents consumed in our research were Alum= AlOH_3 (Alhydrogel 2.0%, Serva Chemical Co., USA), Cholera Toxin (C-3012, Sigma Chemical Co., St. Louis, Mo, USA), Evan's Blue Dye (Merck Chemical Co., Germany), K_3 -EDTA (Sigma Chemical Co., St. Louis, Mo, USA), Phosphate Buffered Saline (Merck Chemical Co., Germany), Rat Histamine kit (LDN Chemical Co., Germany), and Rat Total IgE kit (ICL Chemical Co, USA).

In addition, Encrusted/Crude Peanuts were provided from Safi-Abad Tree-Planting Research Station in the town of Dezful.

Antigen-/Allergen-Preparation

In the present study, PN proteins -as test allergens- were extracted from fresh/crude PNs, according to the reference method [19] which is described briefly, as follows:

Primarily, PN-bodies were pulverized by a mill and subsequently, the resulted paste was defatted by n-Hexane (1:3 v/v, 3 times). Following the separation process, residues were deodorized and dried out via gentle heat-treatment. After that, the obtained flour was mixed with Phosphate Buffered Saline (PBS) (1:10 w/v) and subjected to extraction by shaking overnight at 4°C. Then, the resulted suspension was methodically, centrifuged twice for clarification, as mentioned below:

Firstly: Centrifugation at 3500 r/min. and 4°C for 30 min.

Secondly: Centrifugation at 5000 r/min. and 4°C for 20 min.

Afterwards, the supernatant was additionally, filter-sterilized through 0.45- μ m pore-size sterile syringe filters and lastly, the collected extract was stored as frozen at -20°C until need.

PN-Sensitization/Challenges

Initially, to assure in terms of allergology, the employment of naïve animals concerning the allergen studied, pre-study blood-samples were captured (day #1 of the acclimatization-period, n = 42 Wistar rats).

Subsequently, in the beginning of the sensitization-procedure, 21 Wistar rats were randomly, selected and after a short space, exposed to a three-stage sensitization protocol, every other week (i.e., on days of 8-9 ***** 16-17 ***** 24-25), with crude peanut extract (CPE) according to Roy K, *et al.* prescription [20], with a little adjustment. Each sensitization attempt was arranged in order of two successive days:

On the first days of each week (days of 8, 16 and 24): Oral administration of 1 mg CPE plus 10 μ g Cholera-toxin adjuvant/rat.

On the second days of each week (days of 9, 17 and 25): *Intraperitoneal* (IP) injection of 0.5 μ g CPE plus 0.2 ml Alum adjuvant/rat.

In a parallel manner, naïve/non-sensitized wistar rats (n = 21, as negative counterparts) were studied too, for goal-oriented determinations/resolutions.

Noteworthy, one of the challenging obstacles entangled with actuating the animal models of F.A is the inclination of immune system to develop oral-tolerance as to ingested allergens. Therefore, driving the field-expedient benefits of appropriate adjuvants such as Cholera Toxin and/or Alum, to assist stimulate a Th₂-response, is routine in F.A models [21-28].

Furthermore, in order to provide IgE-antibodies with an occasion of fixing on/binding to effector cells in target organs and in the Meantime, to negate/rule out the presumed/possible (confounding-) pharmaceutical side effects -which would be attributed to adjuvants, all the animals got through with a 1-week length of Rest-Period following the latest sensitizing-dose injection.

Sensitization-Operation Confirmation

Measurement of Total Serum IgE Levels

Subsequently, to a day 32, orbital-plexus blood-samples were obtained by micro-capillary tubes into micro-tubes (1.5 ml in size and 0.75 ml in each one/rat). After 0.5 to 1h coagulation at room temperature, sera were collected. Thereafter, the levels of total serum IgE-immunoglobulins were determined by means of an enzyme immunoassay kit, as described by manufacturer. All analyses were performed in duplicate.

Measurement of Rectal Temperatures

According to procedure, rectal temperatures of the Wistar rats were measured by means of a digital thermometer at the time of study-beginning as well as, one week post sensitization-period following the first *intra-gastric* (*ig*) challenge-dose administration.

Measurement of Plasma Histamine Levels

Conventionally, 25-30 min. after the second *ig* challenging, orbital-plexus blood samples (0.75 ml/rat) were obtained by micro-capillary hematocrit tubes into EDTA micro-tubes for plasma-analysis of histamine. After centrifuging at $2000 \times g$ for 20 min., the plasma-specimens were stored at -20°C until analyzed according to respective brochure, in duplicate.

Assessment of Systemic Anaphylactic Symptoms/Signs

Anaphylactic symptoms and signs of the PN-allergy sensitized wistar rats were evaluated 35-40 min. after the second *ig* challenge-dose gavaging, through the scoring system, which was modified slightly from the earlier prescriptions [18,29,30].

0: No symptoms/signs;

1: Rubbing and scratching around the snout and head;

2: Pilar erecti, puffiness around the eyes and mouth, cringing-humping-hunching, gnashing the teeth, anorexia, diarrhea, urine-incontinence, reduced activity and/or standing-still plus increased respiratory rate;

3: Wheezing, labored respiration, and cyanosis around the mouth and the tail;

4: Symptoms/signs as in No. 3 accompanied by no activity after prodding, lethargy-paralysis or malformation or tremor and convulsions;

5: Death.

Wheal Reaction

2-h before PN-challenge, the abdominal surfaces of wistar rats ($n = 7/\text{group}$), were shaved and used for the ensuing *intra*dermal (*id*) skin-tests with sterile CPE. 5-min. before the test, 100 μl of Evan's blue dye (5 mg/ml.PBS) was injected into the tail-vein of each rat to ease visualize the wheal reaction. Subsequently, 66 μl of the filter-sterilized CPE (3 mg/ml) was administrated intradermally, into the said abdominal skins.

Corresponding measures were carried out using Saline in negative controls, as well. A positive response was defined as a wheal reaction showing up as a blue circle/area greater than 5 mm in diameter while recording at 20 min. post *id*-injection.

Detection of Vascular Leakage

Seven rats from each group received 200 μl of Evan's blue dye (5 mg/ml.PBS) by tail-vein injection, 5 min. before the *intra*peritoneal challenge-dose administration. Subsequently, footpads & paws of the examined animals were scrutinized for manifestations of vascular permeability (visible blue color), 40-45 min. post *ip*-administering of 200 μg of the filter sterilized CPE.

Statistical Analyses

In due time, data were processed by SPSS statistical package, version 19. So, as for serum IgE-antibodies, the differences between two groups were compared *via* Kruskal-Wallis one-way ANOVA and afterwards, by Student's t-test. As to histamine levels, rectal temperatures and anaphylactic scores, the differences were analyzed by one-way ANOVA followed by Mann-Whitney U-test. A probability value of less than 0.05 was recognized to be a significant difference.

Results

Variations in Total Serum IgE Levels

Surprisingly, subsequent to sensitization-period (on day 32), and after the first *ig* challenge-dose administration, the total serum IgE levels had remarkably been elevated overall, in all the sensitized animals (Mean \pm SEM = 348.40 ± 4.86 ng/ml in sensitized vs. 73.67 ± 5.89 ng/ml in non-sensitized subjects; $P = 0.000$ and $n = 21$ Wistar rats/group: Diagram 1).

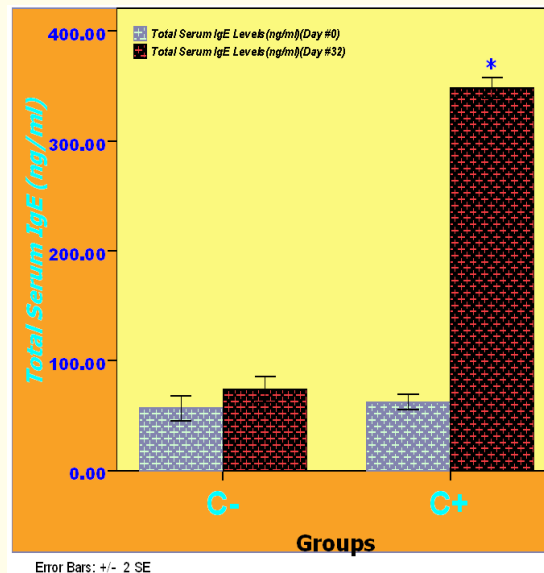


Diagram 1: Levels of PN-induced Total Serum IgE;

Sera from both Groups of Wistar Rats were obtained two times; 1-W pre- and post-sensitization period (On Days #0 and #32). PN-induced IgE levels were determined by using ELISA. Data have been given as Means \pm SEM for each Group.

PN: Peanut, IgE: Immunoglobulin E, W: Week, ELISA: Enzyme-Linked Immunosorbent Assay, SEM: Standard Error of Means, C-: Negative Control, and C+: Positive Control.

Day #0; $P < 0.427$, Negative Control & Positive Control Groups, $n = 21$ Wistar rats per group.

Day #32; * $P < 0.000$, Positive Control Group vs. Negative Control Group, $n = 21$ Wistar rats per group.

Variations in Rectal Temperatures

In a parallel manner, 20-25 min. following the first ig challenge-dose, all the wistar rats in positive control group experienced, predictably, a fall in rectal temperatures of 2 to 4°C 1-week post sensitization period (on day 32, Mean \pm SEM= 34.23 \pm 0.10°C in sensitized vs. 36.64 \pm 0.08°C in non-sensitized animals; $P = 0.000$ and $n = 21$ Wistar rats/group: Diagram 2).

Histamine Release Measurements after PNE-Challenges

Accordingly, as illustrated in diagram #3, 25-30 minutes subsequent to second ig challenge-dose-prescription, the plasma histamine levels had markedly been elevated in positive control group in contrast with negative counterparts (day 32, Mean \pm SEM = 141.15 \pm 10.19 ng/ml in sensitized vs. 10.60 \pm 1.36 ng/ml in non-sensitized/naïve animals; $P = 0.000$ and $n = 21$ Wistar rats/group: Diagram 3).

Anaphylactic Signs/Symptoms Scoring following PNE-Challenges

From a clinical point of view, all the test animals in positive control group manifested typically, the characteristics/features of an anaphylaxis 1-week post sensitization-period and subsequent to second ig challenge-dose administration. On the contrary, none of the naïve/non-sensitized controls developed an anaphylactic-reaction sequelae (day 32, $n = 21$ Wistar rats/group and, median scores = 3 and 0, respectively).

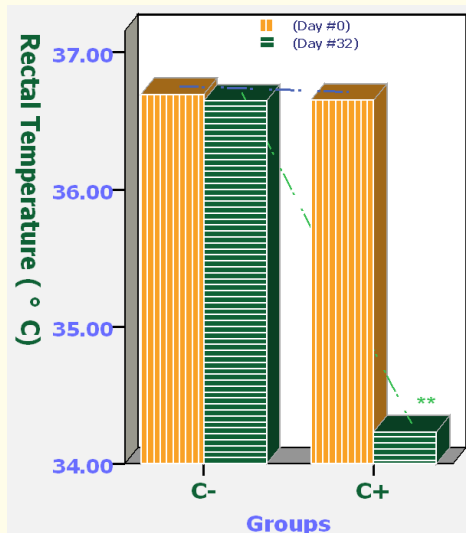


Diagram 2: Variations in Rectal Temperatures of Wistar Rats at the time of study-initiation (On Day #0) and one week post sensitization-period (On Day #32); Data have been given as Means ± SEM for each Group.

Day #0; $P < 0.744$, Negative Control & Positive Control Groups, $n = 21$ Wistar rats per group.

Day #32; $**P < 0.000$, Positive Control Group vs. Negative Control Group, $n = 21$ Wistar rats per group.

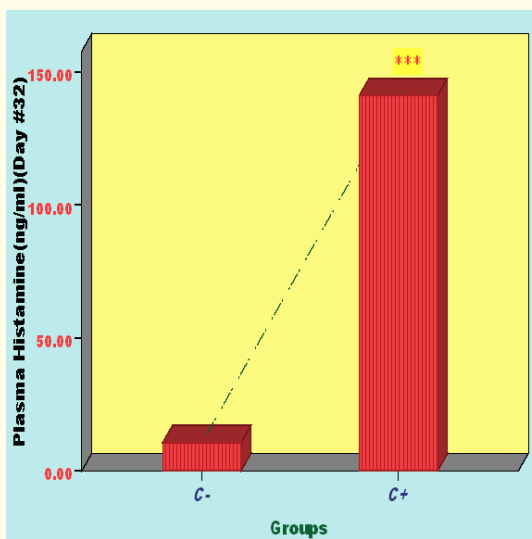


Diagram3: Plasma Histamine Levels 1-W post-sensitization period (On Day #32) followed by ig PNE-challenges in both Groups of the Wistar Rats; Blood specimens for plasma histamine levels were obtained 25-30 min after the second challenge-dose administration and were determined by using ELISA. Data have been given as Means ± SEM for each Group. W: Week, ig: Intra-gastric, PNE: Peanut Extract, ELISA: Enzyme-Linked Immunosorbent Assay, SEM: Standard Error of Means, C+: Positive Control; C-: Negative Control.

Day #32, $***P < 0.000$, Positive Control Group vs. Negative Control Group, $n = 21$ Wistar rats per group.

For a wonder, the sensitized wistar rats had been affected with apparent/undocumented physical malformation, also with, some other novel manifestation -Figure 1.

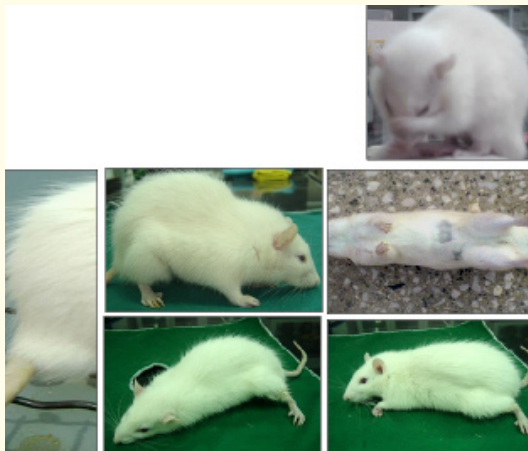


Figure 1: Clinical Signs/Symptoms of Anaphylactic Reactions after ig Peanut Extract-challenges in Wistar Rats under investigation; Wistar Rats in both of Groups (n = 21, each) were challenged intra-gastrically with Crude Peanut Extract one Week following sensitization period (On Day #32). Clinical Signs/Symptoms of Anaphylaxis were evaluated 35-40 min after the second challenge-dose administration.

Statistically, significant differences in anaphylactic symptom-scores between two groups were achieved (P = 0.000: Diagram 4).

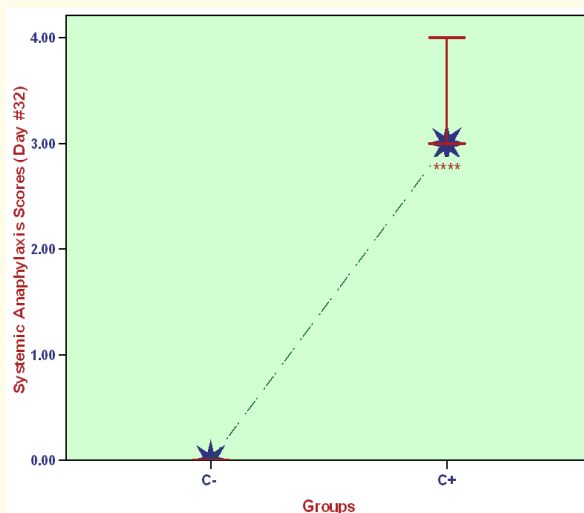


Diagram 4: Systemic anaphylaxis-Scores in Wistar Rats following intra-gastric Peanut Extract-challenges; Data have been shown as Median Scores for each Group. Day #32, ****P < 0.000, Positive Control Group vs. Negative Control Group, Median Scores: 3 and 0, respectively, n = 21 Wistar rats per group.

Wheal Reaction Analysis after PNE-Challenge

Expectedly, subsequent to id challenge-dose injection with sterile CPE, abdominal surfaces of the research animals, merely in positive control group showed up, at record time, a wheal reaction as a blue circle/area close to/greater than one centimeter in diameter on day 32 (n = 7 Wistar rats/group: Figure 2).

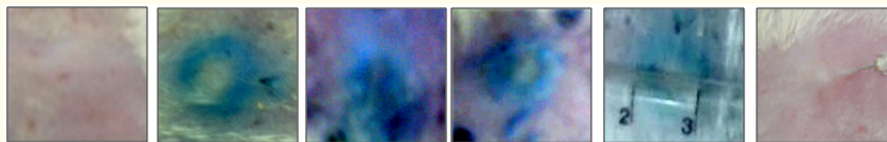


Figure 2: Photographs illustrate Skin Tests 1-W post-sensitization period (On Day #32) and after id CPE-administration; Marked blue-colored Bumps in the Abdominal Skins of the Positive Controls but not in those of Non-sensitized/Naïve Wistar Rats. Results represent 7 Animals from each Group.

Evaluation of Vascular Leakage after PNE-Challenge

Consistently, following ip challenge-dose injection, the footpads and paws of the inspected wistar rats only in positive control group, manifested an extensive vascular permeability -revealed by blue color-, whereas those of the non-sensitized animals had absolutely normal appearances/forms (day 32 and n = 7 Wistar rats/group: Figure 3).



Figure 3: Photographs illustrate Footpads & Paws of Wistar Rats after ip CPE-administration at 1-week post-sensitization period (On Day #32); Marked Vascular Leakage (livid) and Deformity were seen only in the Positive Controls. Results represent 7 Animals from each Group.

Discussion

Sounding the alarm, we say again that the allergic disorders constitute one of the major concerns of modern day medicine. In particular, evident intensification in the incidence of food allergies announces the necessity of additional assessments to enhance any requisite, encountering strengths including preventative and therapeutic strategies in this field.

On the other hand, extensive investigations of humans are restricted moralistically also, considering the risk of likely life-threatening events. This prompts the researchers for exploiting of the pertinent exploratory animals in order to initiate/operate an appropriate action system for food allergies. However, due to a variety of reasons such as idiosyncratic genetic-construct and/or poverty/weakness of the supposed homeostatic similarities between human and the employed laboratory animals (e.g., immune un/responsiveness to particular proteins [31,32] -strictly speaking; Epitopes-), not all of them necessarily, offer hope for desired therapies in human being subjects. Therefore, there is up to now, no perfect model of F.A. Nonetheless, the need for functionally/practically compatible animal models cannot be disputed, never.

Despite few immunological studies denoting various similarities between large animals and human physiology, relatively a few of the reagents/conditions required for studies of allergic disorders in such models are available, including sensitive and specific assays for total and antigen-specific IgEs, etc. Hence, the majority of animal-model studies have been focused on rodent mammals.

Chronologically, Brown Norway rat is known as a sole IgE responder, allowing some level of comparison to atopic/allergic humans [12-17]. Consistently, it is claimed that other rat-strains fail to yield a quantifiable level of IgE-antibodies.

But here, irrespective of the evidence [14], our outcomes/analyses in the current research demonstrated the immunological and clinical (systemic and local) features of the PN allergy as experienced by human beings. In a word, fascinating results obtained in our study signified/suggested that the Wistar rats have been sensitized completely/typically -100% IgE responding. Insofar as, the significant elevation of the PN-induced total serum IgE levels was confirmed overall, in all the PN-sensitized wistar rats ($p = 0.000$, in contrast with negative control animals).

Even Further, as an ideal representative of an anaphylactic shock response, all the sensitized rats incurred a drop in rectal temperatures of 2 to (close) 4°C after the first *ig* challenge-dose administration. Accordingly, plasma histamine levels and anaphylactic-symptom scores in positive control group had a significant increment as compared with negative control group [($p = 0.000$) and ($p = 0.000$), respectively], subsequent to second *ig* challenge dosing -1 mg of CPE/rat; as the first one but, 25-30 min. later.

In a parallel manner, the histamine discharge led to the vascular permeability-expansion as well as, to obvious wheal reactions which are both, referred to as hallmarks of an anaphylactic response. Notably, the sensitized wistars in our study manifested moreover, some undocumented/novel anaphylactic symptoms/signs, including Anorexia, Urine-incontinence, Gnashing, Cringing/Hunching, Physical Distortion -footpads & paws deformity-, and Lethargy/Paralysis. Especially, even though the Death of the laboratory animals is not as usual as it is seen in humans undergoing the anaphylaxis-complications, however, one Death was surprisingly occurred too, in the sensitized Wistar rats in our study -Figure 1; Right/Middle.

So, according to attained rational/convincing findings, of acute allergic skin-test responses, of variations in vascular leakage, of systemic anaphylactic-symptom scores and plasma histamine release measurements after PNE-challenges, as well as, via the completion of other complementary tests, we hereby address strongly, a successful sensitization-induction achievement in the Wistar-strain-rat, for the first time.

Conclusions

So much is certain that our comprehensive understanding of the underlying pathomechanisms of allergies is an urgent issue and doubtlessly, will warrant the search of appropriate approaches that can help managing of these maladies' consequences.

Fundamentally, to improve our comprehension of IgE-antibodies, as well as, for scrutinizing the IgE-mediated hypersensitivity reactions to foods and so forth, there is an urgency to actuate appropriate animal models. Collectively, animal models, as a reliable Means, possess a large quantity of qualifications to cope the problematic complications encompassing the allergies. But, for an animal model to be duly of efficacy in the purview of F.A we need to realize the functional/experimental characteristics of such models and, to identify the respective impediments, in particular, with respect to their feasibility, reproducibility and reliability under different/distinctive situations.

In conclusion, although additional assessments are warranted with refined, weak/strong allergens, and allergenic intact-foods to further validate the improved Wistar model, however, our significant findings in this investigation denoted, in the first place, the IgE-antibodies mediation; Anaphylactic Pathway, in activation of the effector cells in the Wistar rats sensitized orally by PN-allergens. Second, they affirmed/supported the foresaid strain as a fitting/prone model for inspecting/elucidating the PN allergy-pertaining pathophysiological aspects/traits, which eventually will allow finely-founded judgments to be constructed concerning the temperament of possible hazards associated with type-1 hypersensitivity disorders.

At last, it must be noted that having the Wistar rats bred for multiple generations and keeping them naïve/non-sensitized as to the allergen of interest also, the cross-reactive allergenic proteins, might amend the data from our examination, as much.

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