Cell-free Fat Extract Promote Axon Regeneration: a Pre-clinical Study.

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Research

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Abstract

Background: Injured axons of the central nervous system (CNS) hardly regenerate due to their poor intrinsic regeneration ability and adverse microenvironment at the site of injury; previous studies aimed to investigate both the above-mentioned factors and achieved some success. Compared with the gene-editing technology, stem cell therapy has unique advantages, such as low risk of tumor formation and ease-of-use, among others. However, traditional stem cell therapy is hindered by safety and immunogenicity issues. Previously, we developed a cell-free extract directly from human fat tissue (CEFFE) and demonstrated its proangiogenic capacity. Herein, we aimed to evaluate its potential therapeutic effect in injured CNS.

Methods: Therapeutic potential of CEFFE in injured CNS was investigated, using a mouse optic nerve crush model. After the optic nerve was crushed, CEFFE was injected intravitreally. Two weeks post-injury, the number of regenerated axons was measured at different distances from the crush site (250, 500, 750, 1000, and 1500 μm). In addition, surviving retinal ganglion cells (RGCs) were counted. Cellular locations and catalogs of CEFFE were analyzed by gene ontology (GO) annotation. Western blotting (WB) was performed to evaluate the therapeutic effects of CEFFE on the molecular level. An enzyme-linked immunosorbent assay (ELISA) was used to examine the levels of inflammatory factors in CEFFE.

Results: Compared with brain-derived neurotrophic factor (BDNF), CEFFE effectively promoted axon regeneration and RGCs survival. GO annotation showed that 146 proteins were involved in axon genesis or neurogenesis, most of which were located intracellularly. WB and ELISA results showed that CEFFE acted on diverse pathways and involved high levels of inflammatory factors.

Conclusions: The present findings have shown that CEFFE may promote axon regeneration and increase RGCs survival. The levels of factors involved in CEFFE were much higher than those observed in the traditional secretome; CEFFE involves multiple factors and affects several mechanisms. This study has revealeded the potential application of CEFFE for the treatment of CNS injury.

Background

The axon is the structural and functional basis of a neuron, which plays an important role in transmitting signals and substances within the body. After central nervous system (CNS) injury, lesioned axons fail to regenerate, which leads to impaired functional recovery[1, 2] At present, the most commonly used model to mimic axon injury is the optic nerve crush model, as the retina is classified as part of the CNS and the characteristics of its regenerative response are similar to those observed elsewhere in the CNS, including the brain and spinal cord[3, 4]. More importantly, the mechanism of injury has been verified by the optic nerve crush model and translated to other axon injury models, including those of spinal cord injury.

In the last decade, research into axon regeneration has mainly focused on two aspects: abolishing or neutralizing the extrinsic inhibitory molecules associated with glial scars and myelin debris, and altering intrinsic gene expression, such as that of *PTEN*, *KLF*, and *S0X11*, among others [5–7]. Stem cell therapy is the most clinically promising tool in this context. Stem cells have been shown to promote axon regeneration. Among stem cell types, adipose-derived stem cells (ADSCs) are preferred because they are easily available, have low levels of immunogenicity, and have multiple differentiation capacities. Stem cells may play a role in therapy by replacing host cells and secreting trophic factors. However, there is no credible evidence showing that ADSCs can directly differentiate into retinal ganglion cells (RGCs). ADSCs contain many trophic factors, such as the vascular endothelial growth factor (VEGF), brain-

derived neurotrophic factor (BDNF), and nerve growth factor (NGF). Previous studies have shown that ADSCs may affect paracrine secretion.

Applied trophic factors, such as BDNF or NGF hardly affect injured axon regeneration; however, some studies have shown that the combined utilization of trophic factors is better than the utilization of a single factor. We have previously developed a new technology to produce cell-free aqueous human fat tissue extract (CEFFE). In our previous study[8], we have found abundant growth factors (BDNF levels of 1860.99 pg/ml, glial cell line–derived neurotrophic factor (GDNF) levels of 1823.23 pg/ml, TGF-β levels of 1019.72 pg/ml) and revealed their role in angiogenesis. Therefore, we investigated whether the CEFFE can promote axon regeneration. The aim of this study was to test this hypothesis by evaluating the effects of CEFFE on axon regeneration in a mouse optic nerve crush model and to investigate the underlying mechanisms.

Methods

This pre-clinical study investigated the therapeutic potential of CEFFE in injured CNS using an optic nerve crush model.

CEFFE preparation

The CEFFE was prepared in accordance with previously established methods[8–10]. Briefly, human adipose tissue was obtained from healthy female donors who underwent liposuction. The tissue was rinsed with saline to remove red blood cells and centrifuged at 1200 × g for 3 min. The upper oily and lower fluid layers were then discarded, and the middle fat layer was harvested and mechanically emulsified. The emulsified fat was then frozen at -80°C and thawed at 37°C to further disrupt the cells in the fat tissue. After one cycle of the freeze/thaw process, the fat was centrifuged again at 2000 × g for 5 min and separated into four layers, and the third aqueous layer containing the CEFFE was collected and frozen at -80°C for experimental purposes. The final extract was passed through a 0.22-μm filter (Corning Glass Works, Corning, NY, USA) for sterilization and removal of cell debris. The CEFFE protein concentrations were measured, using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The study was approved by the Ethics Committee of the Shanghai Jiaotong University School of Medicine, Shanghai, China.

Surgical procedures

Mice aged 5 weeks were used in this experiment. For all surgical procedures, mice were anesthetized with 1% pentobarbital sodium (10ul/g). After surgery, an ointment containing an ophthalmic antibiotic was applied to protect the cornea. Mice were kept on a 12-h light/dark cycle with free access to food and water. Mice with opaque lenses were excluded from the experiment.

After the mice were anesthetized, the left optic nerve was exposed intraorbitally. The optic nerve was crushed with forceps (Fine Science Tools, 11480-11) for 5 s, 1 mm distal to the eyeball. Care was taken to avoid damaging the underlying ophthalmic arteries. Immediately after crushing, a fine glass micropipette was inserted into the posterior chamber of the left eye to avoid damaging the lens. Before injection, approximately 3 ul vitreous humor was pumped out. Subsequently, using a Nanoliter 2010 injector (WPI, NL2010MC2T), 3 ul of the CEFFE, BDNF, or phosphate-buffered saline (PBS) were slowly injected into the vitreous chamber. The needle was removed after 5 min until the solution completely diffused. And in some experiments, the same volume of CEFFE, BDNF or PBS was injected at the appropriate time.

To label regenerating axons, 2 ul of Alexa-555 conjugated cholera toxin β subunit (CTB) (2 μg/μL, C22843, ThermoFisher) was injected into the vitreous with a Hamilton syringe (80030, Hamilton) two weeks later after injury.

Immunohistochemistry

Mice were humanely sacrificed and intracardially perfused with a cold saline solution, followed by a PBS solution containing 4% PFA. The eyes and optic nerves were carefully removed from the connective tissue, postfixed for 2 h in 4% PFA solution at 4°C, and subsequently transferred to 30% sucrose for 48 h at 4°C. For whole-mount retina staining, the retina was cut into petals and blocked in PBS containing 5% normal goat serum and 0.4% Triton X-100 for 2 h at room temperature. Thereafter, the tissues were incubated with primary antibody-TUJ1 (1:500 dilution, Abcam ab18207) in a blocking buffer overnight at 4°C. After three rinses with PBST, retinas were incubated with the secondary antibody goat anti-rabbit Alex-488 (1:1000 dilution, ThermoFisher R37116). Following three washes with PBST, the tissues were sealed for observation. The optic nerve was embedded in O.C.T. Compound (25608-930, SAKURA) and longitudinal sections (14 μm) were prepared and stored at -20°C until further use.

Quantification of axons and RGCs

The number of CTB-labeled axons was counted at different distances from the crush site (250, 500, 750, 1000, and 1500 μm) in four sections per nerve. Each group included six mice. The cross-sectional width of the nerve was measured at the point at which the counts were taken and used to calculate the number of axons per millimeter of nerve width. The number of axons per millimeter was then averaged over all sections. In addition, Σad, the total number of axons extending distance d in a nerve with radius r, was estimated by summing over all sections with thickness t (14 μm), as follows: $\Sigma ad = \pi r2 \times [\text{average axons/mm}]/t$.

For RGCs counting, whole mount retinas were immunostained with the Tuj1 antibody and six to nine fields were randomly sampled from the peripheral regions of each retina. The percentage of RGCs survival was calculated as the ratio of the number of surviving RGCs in the crushed eyes to that in the contralateral uninjured eyes. Counts of surviving cells and regenerated axons were performed by a single observer who was blinded to the surgical manipulations and treatments.

Proteomics analysis

The CEFFE samples were processed for quantitative proteomic analysis as previously described[8]. Briefly, protein concentrations of CEFFE were measured, using a BCA protein assay kit. The samples were then digested with trypsin for subsequent Liquid chromatography-tandem mass spectrometry analysis. Gene ontology (GO) analysis was performed to classify all identified protein types into three categories (cell component, molecular function, and biological process), using the UniPort-GOA database (http://www.ebi.ac.uk/ GOA/), InterProScan (http://www.ebi.ac.uk/interpro/), and GO annotation (http://geneontology.org/). Proteins related to axon genesis and neurogenesis were also identified.

Western blot assays

Three mice per condition were sacrificed by cervical dislocation and retinas were dissected, homogenized, and solubilized at 4°C in a cell lysis buffer (P0013, Beyotime), supplemented with 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, 1 mM Na3VO4, and a protease inhibitor. Western blotting was performed as previously described. Total protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by western blotting with anti-p-mTOR (Merck SAB5700327, dilution 1:1000), anti-mTOR (Merck SAB2702297, dilution 1:1000), anti-ROCK2 (Santa Cruz sc-398519, dilution 1:1000), anti-CRMP2 (Abcam ab129082, dilution 1:1000), anti-cleaved calpain (Abcam ab92333, 1:1000), anti-p-PTEN (CST 9551, dilution 1:1000), and anti-β-

actin (Beyotime AF5001, 1:1000). Data analysis was performed using the NIH ImageJ software. The mean density of each band was normalized to that of the β-actin signal in the same sample.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays were performed, according to standard protocols, to quantify the levels of transforming growth factor beta 1 (TGFb1), tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), and interleukin 21 (IL-21) in CEFFE.

Statistical analysis

Statistical analysis and graph creation were performed with GraphPad Prism 6 (GraphPad Software). Data were presented as the mean ± standard error of mean. We used analysis of variance with Tukey's test to adjust for multiple comparisons among more than two groups. Differences were considered statistically significant at P-values of < 0.05.

Results

Characterization of CEFFE

The CEFFE was obtained from the lipoaspirate of six healthy volunteers and was produced, as previously described[8]. Approximately 7 ml of pinkish CEFFE was obtained from 50 ml of centrifuged lipoaspirate (collected after the first spin). The total CEFFE protein concentration was 4745.43 ± 751.73 μg/ml (n = 6).

CEFFE promotes axon regeneration

A single injection of the CEFFE had a limited effect on axon regeneration; only a few fibers extended across the crush site. The number of regenerated axons were 1176 at 250 μm from the crush site and sharply dropped to 298 at 500 μm. At 750 and 1000 μm the number was 37 and 0 respectively. Since injured RGCs underwent several pathological phases until death, we adapted our protocol and injected the CEFFE at several key points; as a result, robust axon regeneration was observed (Fig. 1A). The number of regenerated axons at different distances in the CEFFE-thrice-injected group was significantly higher than that in the CEFFE-once-injected group, which were 2417, 1745, 970, 569 and 56 respectively. The number of regenerated axons gradually declined, starting from the lesion site, and the longest regenerated axons reached approximately 1.5 mm from the crush site. Our results revealed that sustained use of the CEFFE can promote axon regeneration.

CEFFE is better than a single trophic factor for axon regeneration

At low concentrations (2 pg/μl) of BDNF, rarely axons extended across the crush site. Meanwhile, high concentrations (5 μg/μl) of the BDNF promoted axon regeneration to some degree. The number of regenerated axons were 1204, 728, 429, 345 and 27 respectively. However, under the same concentration conditions (5 μg/μl), the number of regenerated axons in the CEFFE-treated group was much higher than that in the BDNF-treated group, which were 2408, 1568, 840, 448 and 47. Our results showed that the effect of the CEFFE on axon regeneration was greater than that of a single trophic factor.

CEFFE promotes RGCs survival

In addition to promoting axon regeneration, we found that the CEFFE had a significant protective effect on RGCs survival. In contrast to 14.37% of RGCs surviving in the control group, approximately 47.83% of RGCs survived after the CEFFE injection (Fig. 3). These results indicate neuroprotective effects of the CEFFE.

CEFFE comprises multiple factors related to axon genesis and neurogenesis

To gain a better understanding of CEFFE, we analyzed its components. We have previously reported detailed bioinformatics information (including total protein levels and GO classification) of CEFFE[11]. Herein, we found 146 proteins involved in axon genesis or neurogenesis (Table 1). Among them, only 16 proteins were located extracellularly. Since the regeneration of damaged axons in the CNS may involve the same processes as does axon growth during development[12, 13], we believe that these factors may contribute to axon regeneration and neuroprotection.

Table 1
Protein identified in CEFFE related to neurogenesis or axon genesis

Subcellular localization	Protein description	Gene name	Subcellular localization	Protein description	Gene name
cyto_nucl	Copine-1	CPNE1	cytoplasm	Superoxide dismutase [Cu-Zn]	SOD1
	Dihydropyrimidinase-related protein 2	DPYSL2		Protein S100-B	S100B
	Signal transducer and activator of transcription 3	STAT3		ADP-ribosylation factor 1	ARF1
	Cyclin-dependent kinase 4 inhibitor C	CDKN2C		Tyrosine-protein phosphatase non-receptor type 11	PTPN11
	COP9 signalosome complex subunit 2	COPS2		D-3-phosphoglycerate dehydrogenase	PHGDH
	Platelet-activating factor acetylhydrolase IB subunit alpha	PAFAH1B1		Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	PPP3CA
	Abl interactor 1	ABI1		Vinculin	VCL
	Protein NDRG1	NDRG1		Myc box-dependent-interacting protein 1	BIN1
	Acyl-protein thioesterase 2	LYPLA2		Nucleoside diphosphate kinase B	NME2
extracellular	Laminin subunit beta-2	LAMB2		Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4
	Epidermal growth factor receptor	EGFR		Ras-related protein Rab-10	RAB10
	Mesencephalic astrocyte-derived neurotrophic factor	MANF		Aspartoacylase	ASPA
	Clusterin	CLU		Growth factor receptor-bound protein 2	GRB2
	Integrin beta-1	ITGB1		Mitogen-activated protein kinase 1	MAPK1
	Galectin-1	LGALS1		Protein S100-A6	S100A6
	Fibronectin	FN1		EH domain-containing protein 1	EHD1
	Palmitoyl-protein thioesterase 1	PPT1		Serine/threonine-protein kinase DCLK1	DCLK1
	Laminin subunit beta-1	LAMB1		Ras-related C3 botulinum toxin substrate 1	RAC1
	Beta-2-microglobulin	B2M		ADP-ribosylation factor 4	ARF4

Subcellular localization	Protein description	Gene name	Subcellular localization	Protein description	Gene name
	Thy-1 membrane glycoprotein	THY1		Protein arginine N-methyltransferase 5	PRMT5
	Chondroitin sulfate proteoglycan 4	CSPG4		Pre-mRNA-processing factor 19	PRPF19
	Apolipoprotein D	APOD		Septin-2	SEPT2
	Alpha-N-acetylglucosaminidase	NAGLU		Rho GDP-dissociation inhibitor 1	ARHGDIA
	Mimecan	OGN		Dihydropyrimidinase-related protein 3	DPYSL3
	2',3'-cyclic-nucleotide 3'-phosphodiesterase	CNP		Myosin-10	MYH10
mitochondria	Dynamin-like 120 kDa protein, mitochondrial	OPA1		Src substrate cortactin	CTTN
	2',3'-cyclic-nucleotide 3'-phosphodiesterase	CNP		Cell division control protein 42 homolog	CDC42
	Serine protease HTRA2, mitochondrial	HTRA2		IST1 homolog	IST1
	Versican core protein	VCAN		Dynein light chain 2, cytoplasmic	DYNLL2
	Isocitrate dehydrogenase [NADP], mitochondrial	IDH2		Myotrophin	MTPN
	2-oxoglutarate dehydrogenase, mitochondrial	OGDH		Kinesin-1 heavy chain	KIF5B
	Cytochrome b-c1 complex subunit 8	UQCRQ		Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1
	NIF3-like protein 1	NIF3L1		Calcium/calmodulin-dependent protein kinase type 1	CAMK1
	Trafficking protein particle complex subunit 4	TRAPPC4		Filamin-A	FLNA
	Protein S100-A8	S100A8		Elongation factor 2	EEF2
	Cofilin-1	CFL1		Spectrin beta chain, non-erythrocytic 1	SPTBN1
	Ubiquitin-conjugating enzyme E2 variant 2	UBE2V2		14-3-3 protein epsilon	YWHAE
	Glutathione S-transferase P	GSTP1		Cytoplasmic FMR1-interacting protein 1	CYFIP1
	Apoptosis-inducing factor 1, mitochondrial	AIFM1		Calcineurin subunit B type 1	PPP3R1

Subcellular localization	Protein description	Gene name	Subcellular localization	Protein description	Gene name
nucleus	Stathmin	STMN1		Ras-related protein Rab-21	RAB21
	Protein Hook homolog 3	HOOK3		Biogenesis of lysosome-related organelles complex 1 subunit 2	BLOC1S2
	Serine/threonine-protein kinase PAK 2	PAK2		Transforming protein RhoA	RHOA
	Caprin-1	CAPRIN1		Integrin-linked protein kinase	ILK
	Drebrin	DBN1		Band 4.1-like protein 3	EPB41L3
	Ubiquitin-like modifier-activating enzyme 6	UBA6		14-3-3 protein eta	YWHAH
	Protein enabled homolog	ENAH		Twinfilin-2	TWF2
	PDZ and LIM domain protein 7	PDLIM7		WD repeat-containing protein 1	WDR1
	Echinoderm microtubule-associated protein-like 1	EML1		Protein S100-A9	S100A9
	Vasodilator-stimulated phosphoprotein	VASP		Annexin A1	ANXA1
	PDZ and LIM domain protein 5	PDLIM5		Hypoxanthine-guanine phosphoribosyltransferase	HPRT1
	Dystrophin	DMD		14-3-3 protein gamma	YWHAG
	Transcription factor p65	RELA		Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform	PPP3CB
	EF-hand domain-containing protein D1	EFHD1		Alpha-soluble NSF attachment protein	NAPA
	Spectrin alpha chain, non-erythrocytic 1	SPTAN1		Vesicle-associated membrane protein-associated protein A	VAPA
	Phosphatidylinositol-binding clathrin assembly protein	PICALM		Ezrin	EZR
	Microtubule-associated protein 4	MAP4		Ras GTPase-activating-like protein IQGAP1	IQGAP1
	Microtubule-associated protein 1B	MAP1B		Actin-related protein 2	ACTR2
	Focal adhesion kinase 1	PTK2		Ras-related protein Rap-1A	RAP1A
	Golgin subfamily A member 4	GOLGA4		Mitogen-activated protein kinase 3	MAPK3

Subcellular localization	Protein description	Gene name	Subcellular localization	Protein description	Gene name
	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1		Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	PPP1CC
	Methyl-CpG-binding protein 2	MECP2		X-ray repair cross-complementing protein 5	XRCC5
	Catenin alpha-1	CTNNA1	plasma membrane	Neurotrimin	NTM
	Drebrin-like protein	DBNL		Dystroglycan	DAG1
	Serine/arginine-rich splicing factor 1	SRSF1		Plexin-B2	PLXNB2
	Myotubularin-related protein 2	MTMR2		Integrin alpha-1	ITGA1
	5'-3' exoribonuclease 2	XRN2		Prolow-density lipoprotein receptor-related protein 1	LRP1
	Transcriptional coactivator YAP1	YAP1		Receptor-type tyrosine-protein phosphatase mu	PTPRM
	Reticulon-1	RTN1		Reticulon-4	RTN4
	Microtubule-associated protein tau	MAPT		CD9 antigen	CD9
cytoskeleton	Protein arginine N-methyltransferase 1	PRMT1	endoplasmic reticulum	Endoplasmic reticulum chaperone BiP	HSPA5
				Neuropilin-1	NRP1
				Calreticulin	CALR
				Lysosome membrane protein 2	SCARB2
				C-Jun-amino-terminal kinase-interacting protein 4	SPAG9
				Rab GDP dissociation inhibitor alpha	GDI1
				Beta-hexosaminidase subunit beta	HEXB

CEFFE promotes axon regeneration from diverse aspects

To determine the possible mechanism underlying CEFFE-related axon regeneration, we chose some proteins related to axon regeneration and neuroprotection and examined their expression levels in the retina. Compared with PBS treated group, the expression of mTOR, p-mTOR and CRMP2 were about 1.6, 2.3 and 1.5-fold in CEFFE treated group. While the expression of ROCK2, cleaved-calpain and p-PTEN reduced to 0.5, 0.2 and 0.4-fold. We observed a positive effect of CEFFE on axon regeneration and neuroprotection. In addition, enhancement of inflammation may augment axon

regeneration in the central nervous system[14]. We examined the levels of inflammation factor expression, including TGFb1, TNFα, IL-6, and IL-21, in CEFFE (Table 2), concluding that the CEFFE plays a role in axon regeneration and neuroprotection in different ways.

Table 2
Mean inflammation factor concentration in CEFFE (pg/ml)

	TGFb1	TNFa	IL-6	IL-21
Mean	479.5	48.9	205.2	175.2
SD	216.28	13.49	159.83	125.50

CEFFE, cell-free human fat tissue extract; TGFb1, Transforming growth factor beta 1; TNFα Tumor necrosis factor alpha; IL-6 Interleukin 6; IL-21 Interleukin 21

Discussion

Given our previous findings on CEFFE's positive effects on wound healing, [8–11, 15] we examined its effects on injured CNS repair. In the present study, we demonstrated that CEFFE has neuroprotective and axon regenerating effects on injured RGCs, which suggest its potential clinical applications.

As early as in 1988, R M Lindsay found BDNF could enhance axonal regeneration [16]. In the decades followed, more and more neurotrophic factors such as CNTF, GDNF, and neurotrophin-4/5 etc. were founded to promote axon regeneration [17–20]. However, their use is limited due to the instability of neurotrophic factors in adverse environment associated with CNS injury. In the present study, a single injection of CEFFE did not effectively promote axon regeneration (Fig. 1); multiple injections were required. RGCs death is a gradual process, associated with different characteristics at different stages. As reported previously[21], RGCs underwent a series of changes within 6 h after injury; however, the number of RGCs did not decrease. On days 3–7 post-injury, the rate of RGCs increased gradually, peaking on day 7; accordingly, we injected CEFFE at the corresponding time points; multiple injections of CEFFE promoted RGCs axon regeneration more robustly than did a single injection (Fig. 1).

The use of a single neurotrophic factor may promote RGCs survival or axon regeneration to some extent, as previously reported; however, this method has shown unsatisfactory results in promoting sustained and abundant axon regeneration[22–24]; overcoming these limitations requires further research. Notably, researchers found use of stem cells or signaling inhibitors combined with neurotrophic factors may have a better effect on RGCs survival or axon regeneration than does the use of neurotrophic factors alone.[25–27]. Synergy among neurotrophic factors used is required for effective axon regeneration. The CEFFE is a mixture of diverse proteins, including trophic factors, inflammation factors, and chemokines. Previous studies have shown the role of trophic and inflammation factors in axon regeneration. To further evaluate the effects of the CEFFE, we chose BDNF, one of the most typical trophic factors involved in axon regeneration, as a control. Both CEFFE and BDNF were intravitreally injected on days 0, 3, and 7 post-crush (Fig. 2). The concentration of the CEFFE was approximately 5 ug/ul, which contained 2 pg/ul of BDNF. As we have shown, CEFFE contains diverse neurotrophic factors[8, 15] and may promote axon regeneration that is more robust than that promoted by the use of a single neurotrophic factor; this finding is consistent with those of previous studies.

In addition, the concentration of any single trophic factor in the CEFFE was not very high. Applying the BDNF at the concentration found in CEFFE alone did not promote axon regeneration; meanwhile, the use of CEFFE promoted axon

regeneration effectively. This phenomenon may be accounted for by the synergistic action of neurotrophic factors. Many trophic factors share a common receptor; for example, the NGF binds to the P75 receptor, as does the BDNF. In addition, the BDNF and NT-4/5 share a common receptor, namely, trkB[26, 28] [29, 30]. Various factors may share receptors, activating downstream cascade signals. Concurrently, different factors may act through different signaling pathways, which together may promote axon regeneration. Rho-ROCK and PTEN-mTOR pathways, two signaling pathways that control axon regeneration, were activated by CEFFE [5, 31–33]. As shown in Fig. 4, ROCK2 and PTEN, well-known inhibitors of axon regeneration in the pathways mentioned above, reduced in CEFFE treated group.

The stem cell secretome is defined as a mixture of soluble factors and membrane vesicles, which comprises neurotrophins, microRNAs, and hormones, among others[34, 35]. The secretome from the ADSCs has been used in pre-clinical studies of traumatic brain injury[36–38]. Moreover, in some studies, the secretome from the ADSCs emerged as superior to that from the BMSCs in the treatment of neurodegenerative diseases[39, 40]. Unlike the secretome of the ADSCs, CEFFE includes bioactive extracellular and intracellular components, as shown by our proteomics findings. Some proteins, such as Signal transducer and activator of transcription 3 (STAT3), Ras-related C3 botulinum toxin substrate 1 (RAC1), and cell division control protein 42 homolog (CDC42), have been shown to regulate axon regeneration[41–43]. The concentration of some factors in CEFFE is much higher than that in the secretome from stem cells[44–46]. For example, in the present study, the concentration of the BDNF in CEFFE was approximately 1860.99 pg/ml, while that reported in other studies was approximately 13.22 or 37.03 pg/ml[8, 44, 45]. Inflammatory factors play an important role in promoting axon regeneration[14, 47]. In CEFFE, the concentration of inflammatory factors was higher than that observed elsewhere[44].

Gene editing technology allows to transform the injured neuron into a regenerative state by manipulating a certain gene[5, 13, 48]. Promoting neuronal survival and axon regeneration is among basic strategies to repair injuries in mature CNS. However, although changing intrinsic regulators can affect axon regeneration or neuronal survival, a single gene manipulation may also cause dissonance in axon regeneration or neuron survival, as is the case of *Armcx* and *Sox11*, among others[49, 50]. Our study has shown that the use of the CEFFE may satisfy both conditions, as it reduces the expression of cleaved-calpain that suppresses RGCs apoptosis [51], and the expression of p-mTOR indicated inhibition of autophagy[52] allowing injured RGCs to survive. CRMP2 can stabilize cytoskeletal polymerization, leading to axonal growth[53].

This study has some limitations. From the results we obtained, we prefer to consider CEFFE work in the manner of a whole unit. However, given the large and complex network of factors involved, we could not determine the exact combination of CEFFE factors that affects neuron regeneration. The effects of CEFFE may involve several mechanisms, including activating signaling and pro-inflammatory pathways, and promoting vascularization; we could not determine which of these pathways was the main mechanism of action. Future studies are required to elucidate these mechanisms. Our future research aims to examine the duration of CEFFE action *in vivo* and to optimize its active components for potential clinical applications.

Conclusions

In conclusion, the present findings have shown that CEFFE may promote axon regeneration and increase RGCs survival. CEFFE has several advantages over traditional stem cells. First, CEFFE is a cell-free agent, which reduces its potential immunogenicity and associated genetic concerns, increasing its potential application as an allogeneic biological agent. Second, it is relatively stable, and its ingredients can be quantified, which is convenient for research purposes. Third, the composition of CEFFE is diverse and its components occur at higher concentrations than those observed in the stem cell secretome, which increases its effectiveness.

List Of Abbreviations

CNS, central nervous system

ADSCs, adipose-derived stem cells

RGCs, retinal ganglion cells

VEGF, vascular endothelial growth factor

BDNF, brain-derived neurotrophic factor

NGF, nerve growth factor

CEFFE, cell-free aqueous human fat tissue extract

CTB, conjugated cholera toxin β subunit

GO, gene ontology

TGFb1, transforming growth factor beta 1

TNFα, tumor necrosis factor alpha

IL-6, interleukin 6

IL-21, interleukin 21

PBS, phosphate-buffered saline

Declarations

Ethics approval and consent to participate: The study was approved by the Ethics Committee of the Shanghai Jiaotong University School of Medicine, Shanghai, China.

Consent for publication: not applicable

Availability of data and materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: WZ contributed to conception, study design, and data interpretation. YS and XW contributed to conducting the study, experimentation, and manuscript writing and editing. DC contributed to data collection and analysis. ZY contributed to sample collection and processing. All authors read and approved the final manuscript

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Figures

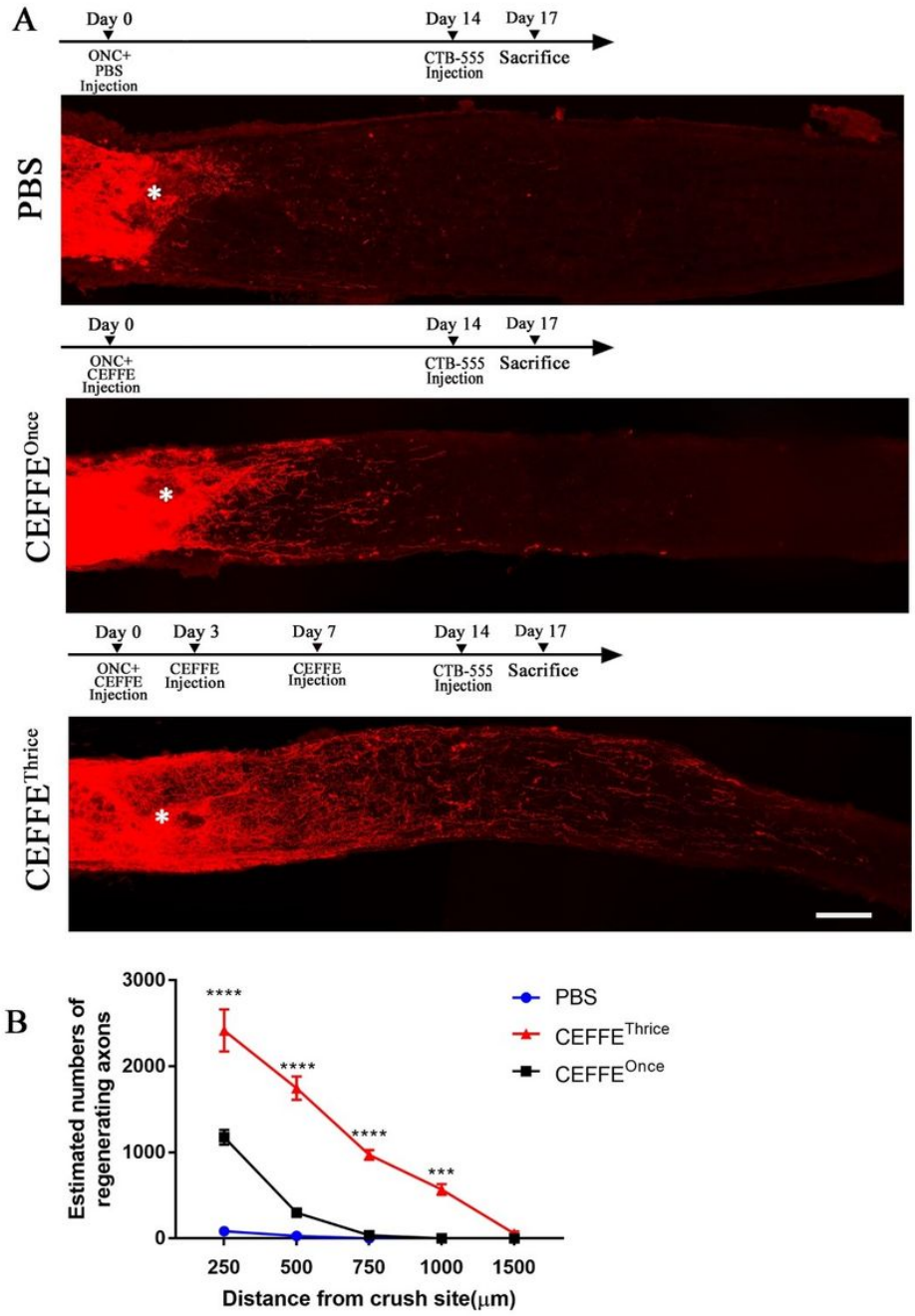

Figure 1

CEFFE promotes axon regeneration. a. Confocal images of optic nerve longitudinal sections showing regenerating fibers labelled with conjugated cholera toxin β subuni-555. *crush site. Scale bar, 100 um. b. Quantification of regenerating axons at different distances distal to the lesion site. ***P<0.001, ****P<0.0001 Data are presented as means ± standard error of mean, n=6 per group. Two-way analysis of variance with Tukey's test. CEFFE, cell-free human fat tissue extract; PBS, phosphate-buffered saline.

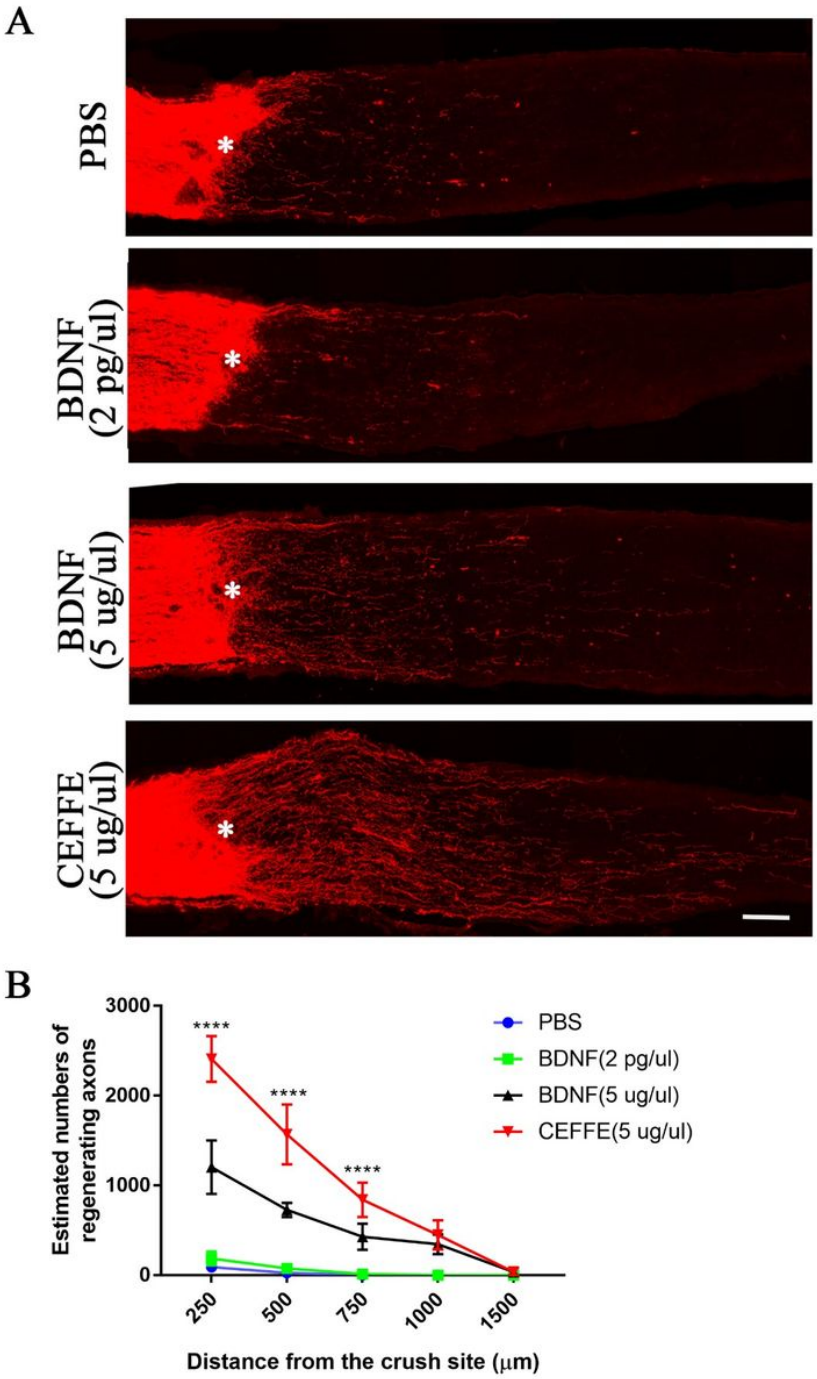

Figure 2

CEFFE promotes axon regeneration that is more robust than that promoted by BDNF. a. Confocal images of optic nerve sections showing regenerating axons labeled by conjugated cholera toxin β subuni-555 2 weeks after optic nerve injury from phosphate-buffered saline (PBS) in BDNF- (2 pg/ul), BDNF- (5 ug/ul), and CEFFE-injected (5 ug/ul) eyes. *crush site. Scale bar, 100 um. b. Quantification of regenerating axons at different distances distal to the lesion site. Data are presented as mean ± standard error of mean, n=6 per group. ****P<0.0001, Two-way analysis of variance with Tukey's test. CEFFE, cell-free aqueous human fat tissue extract; BDNF, brain-derived neurotrophic factor

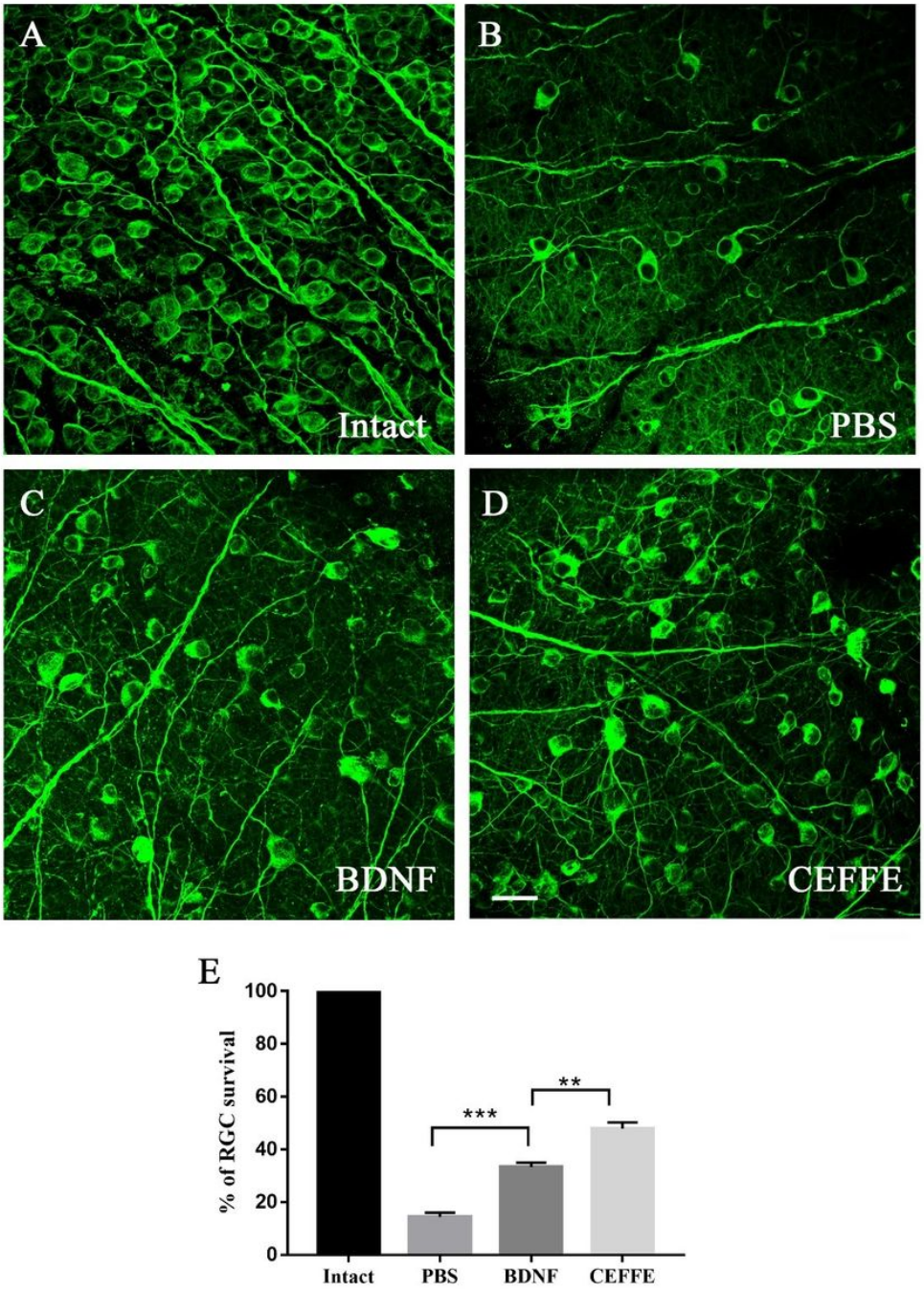

Figure 3

CEFFE promotes retinal ganglion cells (RGCs) survival after optic nerve crush a. Confocal images of retinal whole-mounts show surviving Tuj1+ RGCs at 2 weeks after optic nerve injury. Scale bar, 25 mm. b. Quantification of RGCs survival at 2 weeks after injury, expressed as a percentage of total Tuj1+ RGCs in the uninjured retina. Data are presented as the mean ± standard error of mean, n=6 per group. ****P<0.0001, One-way analysis of variance with Tukey's test. CEFFE, cell-free aqueous human fat tissue extract; BDNF, brain-derived neurotrophic factor; PBS, phosphate-buffered saline

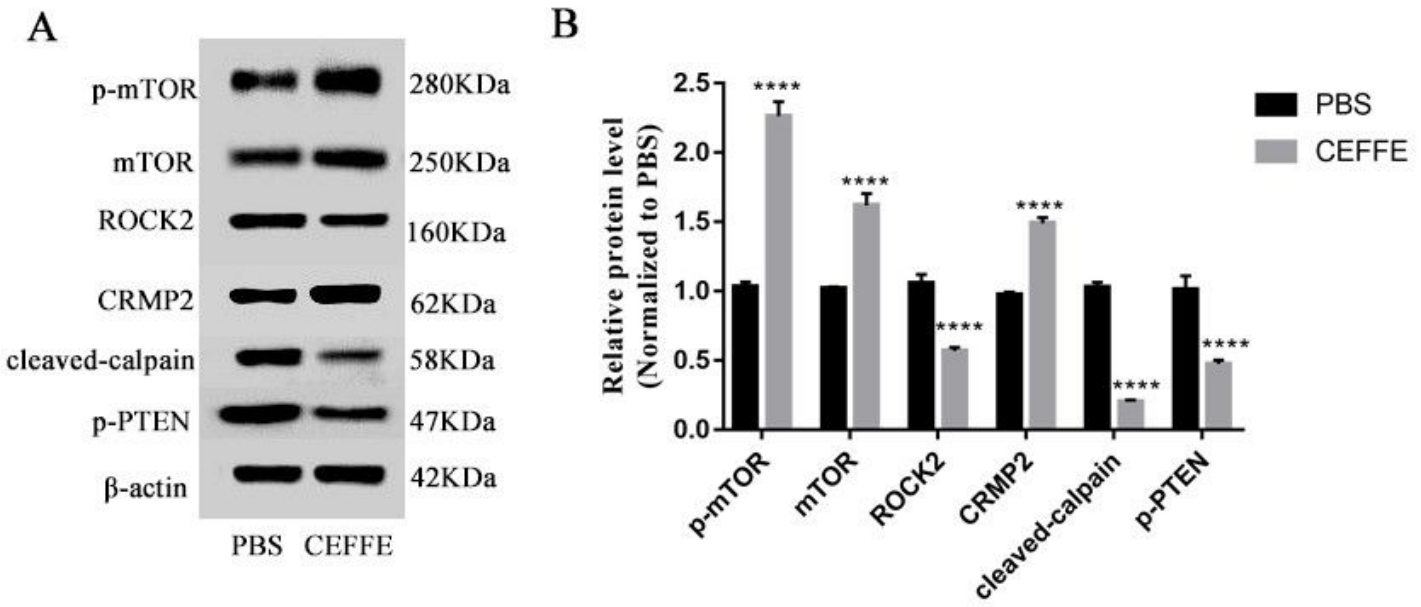

Figure 4

CEFFE modulates apoptosis, autophagy and metabolism a. Western blots of retinal lysates from phosphate-buffered saline (PBS)-treated (con) and CEFFE-treated eyes b. Quantification of band intensities normalized to β-actin as a loading control and relative to that of PBS are depicted (n=3 independent retina; bars represent mean ± standard error of mean, n=6 per group. ****P<0.0001, Two-way analysis of variance with Tukey's test. CEFFE, cell-free aqueous human fat tissue extract